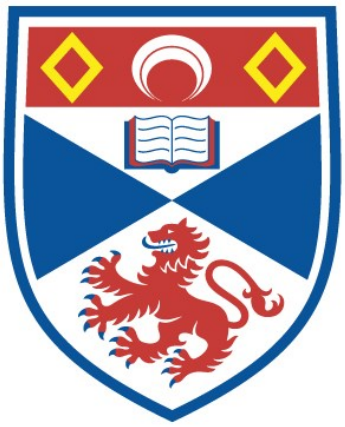
ACTIONS AND DISTRIBUTIONS OF CARDIOACTIVE PEPTIDES IN TWO MOLLUSCS

W. Lesser

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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Submitted for the degree of Doctor of Philosophy

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DECLARATION

I, W. Lesser, hereby certify that this thesis has been composed by myself, that it is a record of my own work, and that it has not been accepted in partial or complete fulfilment of any other degree of professional qualification.

Signed _____ Date 28 Sept 1989

I was admitted to the Faculty of Science of the University of St. Andrews under Ordinance General No. 12 on 7 November, 1985, and as a candidate for the degree of Ph.D. on 9 October, 1986.

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I hereby certify that the candidate has fulfilled the conditions of the Resolution and Regulations appropriate to the Degree of Ph.D.

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ABBREVIATIONS

Amino Acids

A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartic Acid
E	Glu	Glutamic Acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
pQ	Glp	Pyroglutamate
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan
Y	Tyr	Tyrosine

Units of Measure

g	grammes
µg	micrograms
ml	millilitres
µl	microlitres
mm	millimetres
µm	micrometres
nm	nanometres
M	molar
mM	millimolar
µM	micromolar
nM	nanomolar
nmoles	nanomoles
pmoles	picomoles
MΩ	megaohms
ms	milliseconds
mV	millivolts

Abbreviation used in some figures

a	amide

Other Abbreviations

ACh	acetylcholine
4-AP	4-aminopyridine
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(oxy-ethylene-nitilo)tetraacetic acid
FITC	fluorescein isothiocyanate
5-HT	5-hydroxytryptamine
HPLC	high performance liquid chromatography
IBMX	isobutylmethylxanthine
RIA	radioimmunoassay
s.e.m.	standard error of the mean
TEA	tetraethylammonium
UV	ultraviolet

SUMMARY

1. All seven FMRFamide peptides endogenous to Helix aspersa stimulate perfused cardiac ventricles isolated from aestivating Helix. The five heptapeptide amides (pQDPFLRFamide, NDPFLRFamide, NDPYLRFamide, SDPFLRFamide and SEPYLRFamide) are equipotent with each other, but more potent than the two tetrapeptide amides (FMRFamide and FLRFamide).

2. The two endogenous nonapeptide amide SCPs (small cardioactive peptides: SGYLAFPRMamide and MNYLAFPRMamide) are also cardioactive in Helix. They are more potent than the heptapeptides. All nine peptides increase the beat amplitude or beat strength rather than affecting the beat frequency.

3. The potency ratio between the activities of FLRFamide and FMRFamide on ventricles from aestivating Helix is significantly different ($p=0.001$) from that of this peptide pair on ventricles from active animals.

4. RIA analysis of extracts fractionated by HPLC indicate that all nine peptides are present in the circumoesophageal ganglia (brain), the combined visceral and anal nerve trunks, and the anterior aorta.

5. Similar analysis of the heart shows that the tetrapeptides and SCPs are present in the heart; the heptapeptides are absent.

6. Immunohistochemistry reveals a diffuse FMRFamidergic innervation spread evenly throughout the heart. Two clusters of neurones (one each in the left and right parietal ganglia) of the brain also stain positively with FMRFamide antiserum.

7. Calcium-dependent release of the FMRFamide peptides from the brain and the heart has been demonstrated.

8. The perfused cardiac ventricle isolated from Eledone cirrosa is stimulated by the FMRFamide analogues in the reverse order to that of the Helix ventricle; FMRFamide is the most potent, FLRFamide is slightly less potent, and the heptapeptides are the least potent. Six unidentified FMRFamide-immunoreactive peaks are present in the nervous tissue (brain, optic lobes and vena cava); two of these peaks are probably FMRFamide and FLRFamide.

9. The tetrapeptides (but not heptapeptides) reversibly reduce a calcium conductance in the C1 neurone of Helix; this current may be required for transmitter release. This effect may explain presynaptic inhibition by FMRFamide (cf. Haydon & Man-Son-Hing, 1988; Haydon & Zoran, 1989).

CHAPTER ONE

ACTIONS AND DISTRIBUTIONS OF ENDOGENOUS CARDIOACTIVE PEPTIDES IN *Helix aspersa*

Introduction

Cardioregulation in Molluscs

Molluscan cardioregulation has been a source of interest to biologists for at least 100 years. The first experiments were concerned with the effects of electrical current applied to an isolated heart, such as that of the snail (Foster & Dew-Smith, 1875), but interest soon extended to the actions of chemical agents on the hearts of snails and other molluscs (Ransom, 1884).

Molluscan hearts as bioassays. Some molluscan hearts are very sensitive to particular drugs and can therefore be used as bioassays, providing a means of both identification and quantitative estimation of these drugs. For example, the isolated ventricle of the clam *Mercenaria* (formerly *Venus*) *mercenaria* has been used to quantify the acetylcholine (ACh) content of rat brain extracts (Welsh & Hyde, 1944) and to bioassay the 5-hydroxytryptamine (5-HT) content of the mammalian (rat, rabbit and dog) brain (Twarog & Page, 1953). Likewise, the isolated hearts of *Helix* and those of species of *Octopus* and *Eledone* have been employed as bioassays for 5-HT (Erspamer & Ghiretti, 1951).

Molluscan cardioregulators

Acetylcholine, 5-hydroxytryptamine and dopamine. The first three transmitters to be identified as endogenous cardioregulators in molluscs were ACh, 5-HT and dopamine. A summary review of these findings has been written by Welsh (1971). Prosser (1940) suggested that ACh might be an endogenous cardiac inhibitor in *Mercenaria*, as stimulation of the visceral ganglion caused inhibition in diastole resembling the effect of synthetic

ACh. Welsh (1954) used paper chromatography and bioassay to identify and compare the ACh and 5-HT content of Mercenaria mercenaria and Busycon central ganglia. The ratio of the two transmitters was 1:4 in Mercenaria and 1:1 in Busycon. Sweeney (1963) provided fluorometric and paper chromatographic evidence for the presence of dopamine in the ganglia of several molluscan species, and the Tapes watlingi heart is excited (increased beat amplitude and frequency) by low (10^{-7}M) doses of dopamine and inhibited by higher doses (Chong & Phillis, 1965).

Likewise, Kerkut and Cottrell (1963) quantified the ACh and 5-HT content of Helix aspersa brains with chromatography and Helix hearts for bioassay. Their levels were 1-5µg and 0.5-4.0µg respectively per gramme wet weight of tissue. Dopamine is present in the circumoesophageal ring of H. aspersa at 5.5µg/g tissue (Kerkut, Sedden & Walker, 1966) and it increases the beat amplitude of the isolated H. pomatia heart (Osborne, 1970).

Peptides. ACh, 5-HT and dopamine could not account for all the endogenous cardioactive substances in molluscs. Kerkut and Laverack (1958) reported that a fraction of an extract of Helix aspersa brain stimulated the isolated Helix heart. Moreover this extract and 5-HT were pharmacologically and chemically distinguishable (Kerkut & Laverack, 1960). Jaeger (1961, 1966) also detected a cardioactive substance in the snail Strophocheilus oblongus, that was neither 5-HT nor ACh; he suggested it might be a peptide. Frontali, Williams and Welsh (1967) prepared extracts from Busycon canaliculatum and Mercenaria mercenaria and fractionated them on a Sephadex G-15 column. Using the isolated M. mercenaria heart for bioassay, six

peaks of cardioactivity were detected: two inhibitory peaks (ACh and dopamine) and four unidentified excitatory peaks (A, B, C and D according to the order of their elution from the column). When the fractionated extracts of M. mercenaria ganglia, M. mercenaria heart and haemolymph, and Busycon ganglia were assayed and compared, only peak C was common to all tissues. Furthermore, C also seemed the most potent of the four excitatory peaks, so attention was focused upon it. Frontali, Williams and Welsh (1967) suggested that peak C was peptidic in nature.

In 1977, Price and Greenberg purified sufficient quantities of peak C from the clam Macrocallista nimbosa to determine its chemical identity. The central ganglia were dissected from several thousand clams. The active substance was extracted and purified by column centrifugation with a Sephadex G-15 and CM-Sephadex. Electrophoresis, thin layer chromatography and various chemical tests were used to characterise the fractionated extract and the composition of the major component was determined with an automatic amino acid analyser. Controlled enzymic digestion (Edman method) combined with dansylation revealed the sequence Phe-Met-Arg-Phe-NH_2 (FMRFamide).

FMRFamide

FMRFamide-containing species. If peak C generated interest and encouraged research while its active components were still unidentified, the elucidation of the structure and subsequent commercial availability of FMRFamide has been like a spark setting a forest alight. FMRFamide itself has been positively identified in several classes of Mollusca: (1) Polyplacophora: Acanthopleura granulata (Price, Davies, Doble & Greenberg, 1987),

(2) Gastropoda: subclass (a) Prosobranchia: Busycon contrarium, Pomacea paludosa (Price, 1986), (b) Opistobranchia: Aplysia brasiliana (Lehman, Price & Greenberg, 1984), (c) Pulmonata: Order (i) Basommatophora: Helisoma, (Price, Davies, Doble & Greenberg, 1987), Lymnaea stagnalis (Ebberink & Joosse, 1985), Siphonaria pectinata (Price, Cobb, Doble, Kline & Greenberg, 1987), Stagnicola palustris (Price, 1986), (ii) Stylommatophora: Limax maximus (Krajniak, Greenberg, Doble & Price, 1985), Succinea campestris, Cepaea nemoralis (Price, 1986), Strophocheilus oblongus (Price, Davies, Doble & Greenberg, 1987), (3) Cephalopoda: Octopus vulgaris (Voigt & Martin, 1986) and Octopus bimaculoides (Price, 1987) (4) Bivalvia: Geukensia demissa, (Price, 1986) and, of course, Macrocallista nimbosa from which it was originally sequenced (Price & Greenberg, 1977a). This list is not exhaustive, although it suggests that FMRFamide is ubiquitous in molluscs. Moreover, FMRFamide is not the only peptide responsible for the cardioactivity of peak C.

FMRFamide analogues in Helix aspersa. To date, seven analogues of FMRFamide have been identified in Helix alone: one other tetrapeptide amide (Phe-Leu-Arg-Phe-NH_2 [FLRFamide]) and five heptapeptide amides. The sequences of these peptides are listed in table 1.

Phe-Met-Arg-Phe-NH_2	(FMRFamide)
Phe-Leu-Arg-Phe-NH_2	(FLRFamide)
Glp-Asp-Pro-Phe-Leu-Arg-Phe-NH_2	(pQDPFLRFamide)
Ser-Asp-Pro-Phe-Leu-Arg-Phe-NH_2	(SDPFLRFamide)
Asn-Asp-Pro-Phe-Leu-Arg-Phe-NH_2	(NDPFLRFamide)
Asn-Asp-Pro-Tyr-Leu-Arg-Phe-NH_2	(NDPYLRFamide)
Ser-Glu-Pro-Tyr-Leu-Arg-Phe-NH_2	(SEPYLRFamide)

Table 1. The FMRFamide-related peptides of Helix aspersa.

The initial analysis of Helix brain extracts revealed cardioactivity that was not due to FMRFamide. In fact, there was great doubt that FMRFamide itself was present in Helix ganglia. With perseverance, however, not only FMRFamide, but also the heptapeptide analogue pQDPFLRFamide, were identified (Price, Cottrell, Doble, Greenberg, Jorenby, Lehman & Riehm, 1985; Price, Davies, Doble & Greenberg, 1987). FLRFamide and SDPFLRFamide were the next analogues to be sequenced from Helix (Price, Doble, Lee & Greenberg, 1987). The sequencing of NDPFLRFamide (Price, Lesser, Lee, Doble & Greenberg, in prep.) seemed to signal the end of the list of analogues in Helix. Indeed it might well have been, but curiosity and the development and use of a second antiserum (Q2) raised, not against YGGFMRFamide, but against conjugates of pQDPFLRFamide and DDPFLRFamide, revealed two further analogues: NDPYLRFamide and SEPYLRFamide (Price, Lesser, Lee, Doble & Greenberg, in prep.). Unlike the previous three heptapeptide amides to be sequenced from Helix, these latter two have a tyrosine substituted for one of the phenylalanine residues (Phe^4); i.e., an hydroxyl group has been added to the phenyl ring. This seemingly insignificant substitution reduces the affinity of the peptides for the original antiserum (S253) sufficiently that they bind very poorly to S253, and so compete very poorly with the trace (pQYPFLRFamide) in the radioimmunoassay used by Price and his co-workers. Thus the introduction of the Q2 antiserum was absolutely critical to the detection and purification of the last two peptides. The list may still be incomplete.

Interest in FMRFamide has generally been confined to its actions on molluscan muscle or neurones, its effects on the mammalian circulation and central nervous system, and its distribution (determined by immunoreactivity) in the animal kingdom. This latter effort has led to the discovery and identification of still further peptides.

Effects of FMRFamide on molluscan hearts. The myocardial effects of FMRFamide have been well investigated in bivalves, and these actions can be excitatory, inhibitory, or both (depending on the dose) and similar or dissimilar to those of 5-HT (depending on the species) (Painter & Greenberg, 1982).

FMRFamide is a cardioexcitor in Rapana in which it seems to adjust the beat frequency to an approximately constant value (Kobayashi, 1987). FMRFamide increases the cardiac output of Busycon canaliculatum solely by increasing the heart rate as no increase in stroke volume is apparent; but higher doses (10^{-7}M), decrease both the stroke volume and heart rate (Smith & Hill, 1987). When pQDPFLRFamide and FMRFamide were compared on the isolated heart of Helix aspersa, pQDPFLRFamide was approximately one hundred times more potent than FMRFamide (Price, Cottrell, Doble, Greenberg, Jorenby, Lehman & Riehm, 1985).

Effects of FMRFamide on the leech heart. FMRFamide, or a similar molecule, seems to have a cardioregulatory role in the leech Hirudo medicinalis (Kuhlman, Li & Calabrese, 1985a,b; Li & Calabrese, 1987). Immunoreactivity to FMRFamide antisera has been observed in three types of neurones involved in cardio-regulation: motor neurones, modulatory neurones and interneurones which synapse onto the heartbeat central pattern generator

neurones. Application of FMRFamide to the heart or central pattern generator mimics the actions of the innervating neurones. Individual cells that stained positively with the FMRFamide antisera and that innervate the heart have been removed from the leech and their contents analysed by HPLC and subsequent RIA. The results, though inconclusive, suggest strongly that they contain FMRFamide itself. This conclusion is lent credence by the finding of authentic FMRFamide in the polychaete annelid, Nereis virens by Krajniak and Price (1989). Thus, FMRFamide is probably an endogenous cardioregulator in the leech.

Effects of FMRFamide on non-cardiac muscles. Other molluscan muscles respond to FMRFamide. One of these, the Busycon radula protractor muscle, was the primary bioassay used in the identification of FMRFamide (Price & Greenberg, 1977a). FMRFamide induces a slowly developing, but sustained contracture in this muscle; the effect decays slowly in the continued presence of the agonist, but disappears rapidly upon washing (Price & Greenberg, 1980).

At concentrations of 10^{-7}M and greater, FMRFamide induces catch-contractures in the anterior byssus retractor muscle of Mytilus edulis and Geukensia demissa (Painter, 1982); characteristically, the muscles relax only slowly after washing, but rapidly after bath application of 5-HT (Greenberg, Painter & Price, 1981). ACh-induced catch contractures are relaxed by lower (10^{-8}-10^{-7}M) concentrations of FMRFamide (Muneoka & Matsuura, 1985).

Electrically-induced twitch contractions of the Rapana thomasiana radula protractor muscle are potentiated by FMRFamide, though the retractor muscle is unaffected (Yanagawa, Fujiwara,

Takabatake, Muneoka & Kobayashi, 1988). Conversely, FLRFamide potentiates electrically-induced twitch contractions of the retractor muscle, but is without effect on the protractor muscle. In this particular instance, FMRFamide and FLRFamide are similar in their effects, but by acting on antagonistic muscles they are opposing each other. Thus, different components of a single function (radular movement) are allocated to different members of the same peptide family. This is an unusual example of the two tetrapeptides appearing to act at two distinct receptors.

FMRFamide inhibits spontaneous and ACh-induced contractions of the Aplysia californica anterior gizzard, but has no effect on the adenylate cyclase activity of this organ (Austin, Weiss & Lukowiak, 1983).

Effects of FMRFamide analogues on molluscan neurones. The actions and possible mechanisms of action of FMRFamide and some analogues have been compared on identified Helix neurones (Boyd & Walker, 1987; Cottrell & Davies, 1987). These will be discussed in Chapter Three.

Effects of FMRFamide in mammals. In mammals, intracerebroventricular injection of FMRFamide causes an increase in blood pressure on its own and partially blocks [D-Ala^2,Leu^5]enkephalin-induced hypotension (Chai, Tang & Han, 1986). When applied topically, however, it has a vasodilatory effect in the microcirculation (Koo, Chan, Ng & Greenberg, 1983). Antinociceptive actions of morphine are also attenuated by FMRFamide (Tang, Yang & Costa, 1984), as is morphine-induced feeding (Kavaliers, Hirst & Mathers, 1985). These results suggested that many mammalian actions of FMRFamide may be opioid-related.

Immunohistochemistry and the discovery of related peptides. Immunohistochemistry with antisera raised against FMRFamide and its analogues has been a fruitful pursuit. Positive staining has been observed in a range of animals and the references are numerous. One representative survey was conducted by Boer, Schot, Veenstra and Reichelt (1980) in which staining was noted in the central nervous systems of a snail (*Lymnaea stagnalis*), various insects (*Leptinotarsa decemlineata*, *Periplaneta americana*, *Locusta migratoria* and *Pieris brassicae*), the black molly (a fish) (*Poecilia latipinna*) and a mouse. Thus FMRFamide or (more probably) similar peptides are widely distributed throughout the animal kingdom. On its own, however, immunohistochemistry provides little information, only hinting at what might be present. The staining observed must be supported by additional information; the antigens responsible must be identified.

Some workers have identified the source of the observed immunoreactivity with the result that several new peptides have been discovered. For example, LPLRFamide from the chicken has effects on the central nervous system and blood pressure of the rat that mimic those of FMRFamide (Dockray, Reeve, Shively, Gayton & Barnard, 1983). An octapeptide (FLFQPQRFamide, F-8-F-NH_2) and an octadecapeptide (AGEGLSSPFWSLAAPQRFamide, F-18-F-NH_2) have also been sequenced from bovine brain (Yang, Fratta, Majane & Costa, 1985). When injected intracerebroventricularly, the bovine peptides decrease the tail-flick latency in rats, the octapeptide being more potent.

Two octapeptides (Ser-Asp-Arg-Asn-Phe-Leu-Arg-Phe-amide and Thr-Asn-Arg-Asn-Phe-Leu-Arg-Phe-amide) have been sequenced from the pericardial organs of Homarus (Trimmer, Kobierski & Kravitz, 1987); their roles remain to be elucidated. The decapeptide SchistoFLRF-amide (Pro-Asp-Val-Asp-His-Val-Phe-Leu-Arg-Phe-amide) has been sequenced from Schistocerca gregaria (Robb, Packman & Evans, 1989) and is a potent cardioinhibitory agent on the semi-isolated heart of the locust and potentiates contractions of the extensor-tibiae muscle at low concentrations while producing a variable biphasic response at higher concentrations.

AF1 (Lys-Asn-Glu-Phe-Ile-Arg-Phe-amide) from the nematode Ascaris suum (Cowden, Stretton & Davis, 1989) inhibits locomotory movements of intact Ascaris, blocks slow oscillatory potentials and reduces the input resistance of inhibitory neurones at the cellular level.

A family of coelenterate peptides is being revealed with the aid of antisera raised against RFamide, though the initial findings were generated with FMRFamide antisera (Grimmelikhuijzen, Dockray & Schot, 1982). This peptide family includes Antho-RFamide (<Glu-Gly-Arg-Phe-NH_2), (Grimmelikhuijzen & Graff, 1986), Antho-RWamide I (<Glu-Ser-Leu-Arg-Trp-NH_2) and Antho-RWamide II (<Glu-Gly-Leu-Arg-Trp-NH_2) (Graff & Grimmelikhuijzen, 1988a,b) and Pol-RFamide (<Glu-Leu-Leu-Gly-Gly-Arg-Phe-NH_2) (Grimmelikhuijzen Hahn, Rinehart & Spencer, 1988). Generally, these peptides seem confined to neural elements (according to immunohistochemical evidence), and they may act as neurotransmitters or neuro-modulators of slow muscles in coelenterates (McFarlane, Graff & Grimmelikhuijzen, 1987).

SCPs

Their partial purification from Helix. Almost simultaneously with the elucidation of the structure of FMRFamide, Lloyd (1978a,b) was investigating four peaks of cardiac activity from Helix aspersa: an inhibitory peak 2 (assumed to be ACh), and three excitatory ones. Peak 4 appeared to be 5-HT, but peaks 1 and 3 were not due to any of the then known cardioactive substances. The activities of peaks 1 and 3 were destroyed by proteolytic enzymes suggesting that they were due to peptides. Peak 1 could be extracted from the haemolymph, atrium and sub-oesophageal ganglia, but not the cerebral ganglia or ventricle. Gel filtration suggested a molecular weight in excess of 1500 daltons, so the active component was named large cardioactive peptide (LCP). The distribution of peak 3 was more restricted (being confined almost completely to the nervous system), and its activity was stable to heat, acid and alkaline treatment. As its apparent molecular weight was less than 800 daltons, it was named small cardioactive peptide (SCP).

The presence of LCP in the haemolymph at levels sufficient to excite the heart suggested a neurohormonal role, perhaps even including cardioregulation. Despite the ability of SCP to stimulate the isolated heart, the apparent restriction of its distribution to nervous tissue led Lloyd to doubt that it had any physiological role to play in cardioregulation.

The change to Aplysia. Possibly despairing of Helix (other than using its heart for bioassay), Lloyd (1982) turned his attentions to Aplysia. Like Helix, Aplysia also contained non-classical cardioactive substances. The peak equivalent to

LCP was missing, but the SCPs (now two distinct peaks) were present. However, their identity with the SCP fractions of Helix has never been demonstrated.

Structures of SCP_A and SCP_B and their cardiac effects in Aplysia. Sufficient quantities of the SCPs were finally extracted from Aplysia for their structures to be determined. SCP_B was first extracted from the central nervous system of A. brasiliana; and has the sequence Met-Asn-Tyr-Leu-Ala-Phe-Pro-Arg-Met-NH_2 (MNYLAFPRMamide) (Morris, Panico, Karplus, Lloyd & Riniker, 1982). The gene encoding the precursor molecule in Aplysia californica was sequenced next (Mahon, Lloyd, Weiss, Kupfermann & Scheller, 1985). The precursor protein contained one copy of SCP_B; an adjacent sequence was assumed to be SCP_A. Only one copy of each was present. Lastly, SCP_A (Ala-Arg-Pro-Gly-Tyr-Leu-Ala-Phe-Pro-Arg-Met-NH_2 (ARPGYLAFPRMamide) was extracted from the gut of A. californica, confirming the prediction from molecular genetics (Lloyd, Kupfermann & Weiss, 1987). Both peptides are equipotent and appear to act through the mediation of cAMP (Lloyd, Kupfermann & Weiss, 1985) on the isolated Aplysia heart (Cawthorpe, Rosenberg, Colmers, Lukowiak & Drummond, 1985).

Distribution of SCPs in several molluscan species. Biochemical and immunohistochemical studies in Aplysia indicate that the SCPs are found throughout the nervous system, particularly the buccal ganglia where the concentration is ten-fold greater than in other ganglia; they are present in equimolar ratios (Lloyd, Mahon, Kupfermann, Cohen, Scheller & Weiss, 1985).

SCP-immunoreactivity has also been noted in the central nervous system (including a buccal neurone with an axon in the gastro-oesophageal connective) in several other opistobranchs (Longley & Longley, 1985). B11 and B12 (two large buccal neurones in the nudibranch Tritonia) contain SCP in sufficient quantity for extraction and bioassay (on the Helix heart) (Lloyd, 1982) and identification as SCP_B (Willows & Lloyd, 1983). Not surprisingly, the Tritonia gut also responds to SCP (Lloyd, 1982).

SCPs and feeding. The association of the SCPs with feeding behaviour has been a basis of much research, overriding the dwindling interest in a possible cardiovascular role. SCP_A was even sequenced from Aplysia gut extracts (Lloyd, Kupfermann & Weiss, 1987)! Interest has so far been confined to several molluscs: Helisoma trivolvis (Murphy, Lukowiak & Stell, 1985), Tritonia diomedea (Lloyd & Willows, 1988; Willows, Lloyd & Masinovsky, 1988), Limax maximus (Prior & Watson, 1988), not to mention Aplysia (Lloyd, Kupfermann & Weiss, 1984).

Bath application of SCP_B stimulates the patterned motor activity of neurones B5 and B19 in isolated buccal ganglia preparations of Helisoma. B5 innervates the gut and B19; B19 innervates a supralateral radular tensor muscle. Although B5 and B19 do not appear to stain with SCP_B antiserum, other neurones in the buccal ganglia do, and one or more of them may synapse onto B5 or B19.

In Tritonia two SCP_B-containing neurones (B11 and B12) appear to synapse (though perhaps not directly) onto a third neurone, B5, intracellular stimulation of which elicits contractions of the buccal mass inducing swallowing. Electrical

stimulation of B11 enhances the activity of B5 and, itself, induces a backwardly-directed peristalsis of the foregut compatible with swallowing. Stimulation of B12 inhibits the output of B5 and induces a forwardly-directed contraction of the foregut, which would push food toward the mouth and so is not compatible with swallowing. Superfusion of the isolated gut with SCP_B enhances peristalsis in like manner to stimulation of B11. Thus it is quite possible that B11 releases SCP_B onto the anterior gut which responds with peristaltic movements.

SCP_B can initiate or lower the threshold for initiation of the feeding motor program of Limax. However SCP_B has no effect on this program once it has been initiated. SCP_B also increases the burst frequency of the fast salivary burster neurone when bath applied to the isolated central nervous system. Many buccal neurones are immunoreactive to SCP_B antiserum.

SCP_B is present in the buccal ganglia and the nerve that innervates the accessory radula closer muscle of Aplysia. At nanomolar concentrations SCP_B potentiates contractions of this muscle elicited by nervous or chemical stimulation. The potentiation appears to be mediated via cAMP as SCP_B increases the levels of cAMP in this muscle.

Thus, SCP_B appears to initiate or enhance muscular activity associated with feeding in several molluscan species. Moreover, these species also display SCP_B immunoreactivity in their buccal ganglia and its associated musculature. Therefore, SCP_B probably plays an important modulatory role in feeding in molluscs.

Ultrastructural localization, transport and release of SCPs. Immunoelectron microscopy of Aplysia central ganglia showed the SCP immunoreactivity was confined to dense-core vesicles (Cropper, Lloyd, Reed, Tenenbaum, Kupfermann & Weiss, 1987). Transport (Lloyd, 1987) and release (Lloyd, Schacher, Kupfermann & Weiss, 1986) of the peptides has also been demonstrated in Aplysia.

Co-localization of SCPs with other transmitters. The SCPs can be present in cells with other transmitters such as ACh (Lloyd, Mahon, Kupfermann, Cohen, Scheller & Weiss, 1985) and FMRFamide (Lloyd, Frankfurt, Kupfermann & Weiss, 1985).

Post- and pre-synaptic facilitation. The SCPs can mediate both postsynaptic (Lloyd, Kupfermann and Weiss, 1984) and presynaptic (Abrams, Castellucci, Camardo, Kandel & Lloyd, 1984) facilitation. The former process involves raising cAMP levels within the accessory radula closure muscle of Aplysia, thereby potentiating contractions; this effect is similar to that of 5-HT. Presynaptic facilitation by SCPs is also 5-HT-like; it involves a cAMP-dependent closure of a 5-HT-sensitive potassium channel, thereby broadening the presynaptic action potential and facilitating transmitter release.

SCPs of Helix. At long last, Price (1987) returned to the neglected Helix aspersa armed with a rabbit polyclonal antibody against SCP_B from H. Morris (Imperial College, London). After several attempts he was able to extract and purify sufficient peptide for amino acid analysis. On speculation more than hard evidence, he hypothesised the possible sequence: Ser-Gly-Tyr-Leu-Ala-Phe-Pro-Arg-Met-NH_2 (SGYLAFPRMamide). This

sequence has since been confirmed, as has the presence of SCP_B (MNYLAFPRMamide) in Helix (Price, Lesser, Lee, Doble and Greenberg, in prep).

With this information at hand, we now turn to the actions and distributions of these peptides (listed in Table 2) in the common garden snail, Helix aspersa.

FMRFamide-related peptides

Phe-Met-Arg-Phe-NH_2	(FMRFamide)
Phe-Leu-Arg-Phe-NH_2	(FLRFamide)
Glp-Asp-Pro-Phe-Leu-Arg-Phe-NH_2	(pQDPFLRFamide)
Ser-Asp-Pro-Phe-Leu-Arg-Phe-NH_2	(SDPFLRFamide)
Asn-Asp-Pro-Phe-Leu-Arg-Phe-NH_2	(NDPFLRFamide)
Asn-Asp-Pro-Tyr-Leu-Arg-Phe-NH_2	(NDPYLRFamide)
Ser-Glu-Pro-Tyr-Leu-Arg-Phe-NH_2	(SEPYLRFamide)

SCPs

Met-Asn-Tyr-Leu-Ala-Phe-Pro-Arg-Met-NH_2	(MNYLAFPRMamide)
Ser-Gly-Tyr-Leu-Ala-Phe-Pro-Arg-Met-NH_2	(SGYLAFPRMamide)

Table 2. The endogenous cardioactive peptides of Helix aspersa.

Methods and Materials

An Explanation of the Principles Underlying HPLC and RIA

Fractionation of the extracts by HPLC. In the studies reported here, HPLC (High Performance Liquid Chromatography) was used to separate the peptides extracted en mass from the various tissues; to then identify these peptides, one of three antisera (to FMRFamide or SCP_B) was used in the appropriate RIA (RadioImmunoAssay).

The peptides were extracted from the tissues in acetone and the extract loaded onto an HPLC column. According to the solvents and gradients employed, the individual peptides will be retained on the column for varying lengths of time and will thus elute in sequence. The order of elution and the approximate elution time for each peptide can be determined by loading standards (i.e. known quantities of known peptides) on to the column, and running the same gradient after all the extracts or samples have been run. Although the order of elution remains constant as does the approximate time of elution of each peptide, the detection times for the standards do not necessarily correspond exactly to the elution times of the extracts. In the studies reported in this thesis, there were two reasons for the differences in the detection times of standards by HPLC and extracts by RIA. First, the baseline level of UV absorbance established before each run rose and fell markedly when the sample was loaded onto the column. Only after the absorbance had returned close to the baseline, was the loading judged to be complete, and the elution gradient initiated. As this judgement was subjective, and individual samples varied in their UV

absorptions, the elution gradient could not be started at exactly the same time for each run. Secondly, there was a time lag (30-60 seconds) between the monitoring of an absorbance change and the delivery of the responsible drops of eluate to the collecting vials.

The net result of these two factors is that the order of elution of the peptides from a particular column run with a particular gradient remains constant, but the exact time of elution of a particular fraction varies slightly from run to run. Therefore, the relative positions of the various peaks of immunoreactivity are of greater value in evaluating the occurrence of peptides in a sample than their exact elution times.

Detecting the peptides in the fractions by RIA. RIAs are based on the ability of an antigen to displace a radioactive trace bound to an antibody. The degree of displacement can be monitored by counting the radioactivity of either the supernatant, or the sediment, depending on the exact protocol employed.

Three polyclonal antisera raised in rabbits were used in the RIA. The first two (designated S253 and Q2) were produced by D. A. Price (Whitney Laboratory, University of Florida). S253 was raised against a conjugate of YGGFMRFamide. Q2 was initially raised against a conjugate of pQDPFLRFamide, but the rabbit was boosted with a conjugate of DDPFLRFamide. The third (SCP_B) was raised against SCP_B and supplied by H. R. Morris (Imperial College, London).

The protocol described here for the extraction and identification of the peptides using HPLC combined with RIA was developed in the laboratory of D. A. Price; earlier versions have been

described previously (Price, Cobb, Doble, Kline and Greenberg, 1987). The HPLC protocol in use here is the simplest in use at the Whitney Laboratory.

Methods

Preparation of extracts and analysis by HPLC and RIA.

The desired tissue (heart, brain, nerve trunks or aorta) was dissected from Helix aspersa, weighed and immersed in four times its mass of acetone, and kept at -20°C overnight. Usually, tissue from ten animals was used, but five animals were sufficient for the cerebral and suboesophageal ganglia. The atrium to the initial millimetre or so of aorta was removed from the snail and analysed as heart tissue. The suboesophageal ring was removed and analysed as the brain. The nerve trunks were dissected free as long as they remained free from other organs (so that there was no possibility of contamination by other tissue). Thus not all the nerve trunk from a particular animal was analysed for its peptide content but rather approximately half the nerve tissue was used. Similarly, approximately half the length of aorta from the suboesophageal ring was removed as the half closer to the heart was more closely connected to other tissue. The aortas were flushed with saline before their immersion in acetone to remove any blood within them.

After 12-18 hours of incubation, the extract was poured off the tissue, which was then rinsed in several more volumes of acetone; the extract and rinse were pooled. The acetone was evaporated off with an air stream. The remaining liquid was diluted with HPLC buffer A (aqueous 0.1% trifluoracetic acid (TFA)), and the liquid centrifuged at 1000 times gravity to

sediment any particles. The supernatant was loaded onto a reverse-phase HPLC column (Aquapore RP-300, 220 x 2.1mm, 7µm). The column was washed with buffer A, the flow switched to 20% buffer B (80% acetonitrile with 0.1% TFA), and a gradient started to 50% buffer B over 30 minutes. The flow rate was 0.5ml/minute throughout, and 0.5 minute (0.250ml) fractions were collected. Aliquots (2µl or 5µl) were taken from each fraction. RIA buffer (10mM $Na_2HPO_4.7H_2O$, 0.9% NaCl, 25mM EDTA, 0.1% merthiolate, 1% BSA in distilled water, pH 7.5) was added to give a total volume of 50µl. Antiserum (100µl: Q2 at 1:500; S253 at 1:10,000; or SCP_B at 1:10,000) and 100µl trace (^{125}I-pQYPFLRFamide with Q2 and S253, or ^{125}I-SCP_B with SCP_B) were both diluted in RIA buffer, and added to each tube. Standard curves were made from twofold serial dilutions of the appropriate peptide: FMRFamide was used with S253, FLRFamide with Q2, and SCP_B with SCP_B. Thus the contents of the sample tubes could be quantified by comparing their levels of radioactivity with those of the tubes containing known amounts of the appropriate peptide and antiserum. The tubes were then incubated overnight (a minimum of 12 hours) at 4°C. On the following day, 1ml dextran-coated charcoal solution (2.14g $Na_2HPO_4.7H_2O$, 0.28g $NaH_2PO_4.H_2O$, 0.1g merthiolate, 2.5g charcoal, 0.25g dextran per litre water, pH 7.6) was added to each tube. After 10 minutes of incubation, the tubes were spun at 1000 x gravity for 10 minutes at 20°C. The supernatant was pipetted or decanted off and counted in a gamma counter (LKB Model 1275 Minigamma).

The distribution of immunoreactivity within the heart and nervous system determined by wholemount immunohistochemistry.
The hearts or circumoesophageal rings (brains) were dissected from fresh snails. The tissue was pinned out on a Sylgard base in a solution containing paraformaldehyde and picric acid (20g paraformaldehyde, 150ml 0.1% picric acid solution in 1 litre 0.1M phosphate buffer [2.6g $NaH_2PO_4.H_2O$, 11.5g Na_2HPO_4 in 1 litre distilled water, pH 7.5]). The nerve tissue was desheathed almost to the last layer and excess tissue removed from around the other organs. The hearts were split open longitudinally and repinned. The fixative in which the finer dissection had taken place was replaced with clean solution. The dissection dish was covered with Parafilm and kept at $4^{o}C$ overnight (a minimum of 12 hours).

On the following day the fixed tissue was washed in 80% ethanol (6 solution changes at 10 minute intervals), then dehydrated through ethanol (95, 100, 100%) to xylene (50/50 with ethanol, 100, 100, 100, 50/50) and rehydrated (100, 100, 95, 80, 70, 50% ethanol) to phosphate buffered saline (PBS: 10mM $Na_2HPO_4.7H_2O$, 0.9% NaCl in distilled water, pH 7.5). The solutions were changed at 30 minute intervals.

The tissue was then transferred to a blocking solution (10% normal goat serum in PBS^{+} (0.01% merthiolate, 0.25% Triton X-100 in PBS), and placed on a shaker in the dark at $4^{o}C$ for up to 12 hours. The tissue was then transferred to the primary antibody solution (S253 at 1:200 diluted in blocking solution)

for 48 hours, washed in PBS^+ for 24 hours (with 15 changes of solution), incubated in goat-anti-rabbit antiserum conjugated to FITC (diluted 1:50 in blocking solution) for 24 hours, washed for 24 hours in PBS^+ with 15 changes, and mounted in carbonate-buffered glycerol (1 part 0.5M $Na_2CO_3/NaHCO_3$: 2 parts glycerol, pH 8.6).

The mounted tissues were viewed under epifluorescence with a Nikon microscope with 460 - 485nm excitation wavelength and 510nm cut-off wavelength. Photographs were taken with a Nikon camera equipped with a Nikon automatic exposure meter; Ektachrome 400 ASA film. (Method modified from Lehman and Price (1987).)

Controls for specificity of staining. Some ganglia were treated as usual but incubated with primary antiserum which had been preincubated with 10^{-6}M FMRFamide for 24 hours. Only background fluorescence was observed when these ganglia were viewed under epifluorescence.

Calcium-dependent release of the FMRFamide peptides

The heart. The heart was cannulated at the aortic end of the ventricle. The aorta was cut distal to the insertion of the cannula, and the atrium cut at its junction with the vein. The cannula was connected to a loop system, devised by K. Payza (1987), but modified as follows. The dead space of the loop was reduced to a minimum by connecting the three-way taps directly to the Y-tubes - especially at the sample loop, so that a sharp front of saline would enter the heart (Fig. 1). The sample loop had a volume of 1.8ml. The perfusion rate was set very low (20µl/minute).

The heart was first perfused with a normal saline (7mM $CaCl_2$, 4mM KCl, 5mM $MgCl_2$, 80mM NaCl, 5mM TrisHCl, pH 7.5)

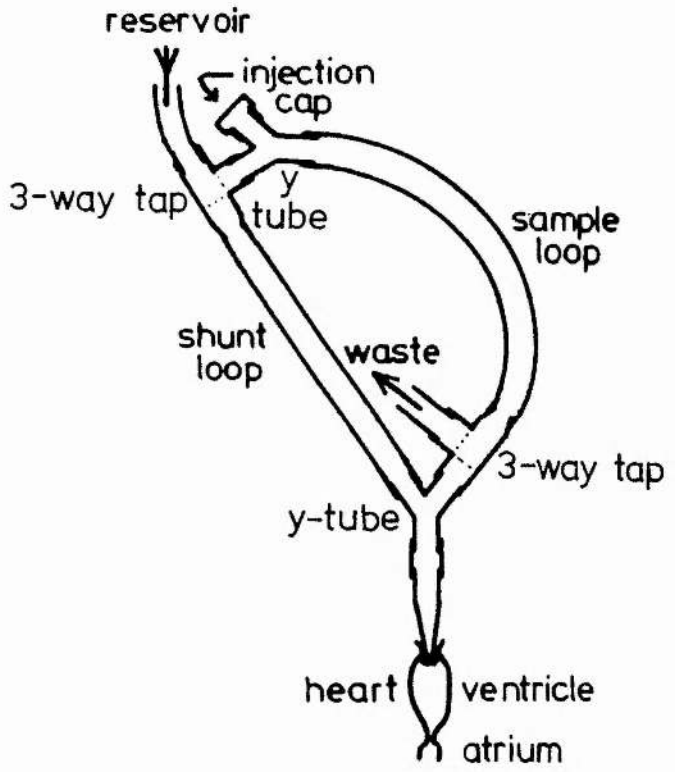


Figure 1. The closed loop system for the stimulated release of FMRFamide peptides from the isolated heart (shown) and circumoesophageal ring (not shown) of Helix (modified from Payza, 1987). When the taps are adjusted to prevent saline flowing across the broken (dashed) lines in the three-way taps, the saline flows through the shunt loop and the desired saline can be injected into the sample loop through the injection cap, displacing the saline already present which flows out the waste tubing. If the taps are then adjusted so that flow across the dotted lines is obstructed, then the contents of the sample loop will flow through the heart. Note the flow through the heart is from the ventricle to the atrium. This allowed the atrium, ventricle, and atrial-ventricular junction all to be exposed to the salines and thus to contribute to the release of peptides.

for 30 minutes to allow the heart to equilibrate with its new conditions and to wash away blood and any free enzymes or other proteins. Drops of perfusate were then collected in sequence, one drop per tube. After five drops had been collected, the sample loop was filled with 0mM $CaCl_2$/40mM KCl saline (the calcium was replaced by magnesium and sodium was replaced by potassium to keep the monovalent ion and divalent ion totals constant), and twenty to thirty more drops were collected. The saline was then returned to its starting composition and a further twenty to thirty drops were collected. In normal saline, the heart presumably repolarised and the calcium levels were restored. Finally, the heart was perfused with 7mM $CaCl_2$/40mM KCl saline (i.e., normal calcium, high potassium) and twenty to thirty drops collected.

At the end of the experiment, each tube was assayed by RIA, each 40µl drop serving as a sample; i.e. 100µl ^{125}I-pQYPFLRFamide trace and 100µl S253 antiserum were added to each tube, and the assay carried out as described above.

The circumoesophageal ring. The artery supplying the buccal mass was ligated and the middle foot artery (Pentreath & Cottrell, 1970) cannulated. After the remaining vessels and nerves had been cut as distal to the circumoesophageal ring as possible, the ring was removed and hung from its cannula in a manner similar to the heart. The protocol was somewhat different to that for the heart. First, the perfusion rate was increased to 40µl/minute. Also, the order of the perfusing solutions, after the 30 minute equilibration and rinsing period was: 7mM

$CaCl_2$/4mM KCl; 2mM $CaCl_2$/4mM KCl; 2mM $CaCl_2$/40mM KCl; 7mM $CaCl_2$/4mM KCl; and 7mM $CaCl_2$/40mM KCl. As with the heart, twenty to thirty drops were collected for each condition, except for the initial saline, where only 5 drops were collected. The tubes were assayed by RIA, as for the heart.

Several points became apparent as this protocol was developed. The total monovalent and divalent ion concentrations had to remain constant. Altering them, especially the divalent ion total, affected the sensitivity of the RIA. A similar observation was made by Carroll, Carrow and Calabrese (1986). The use of TrisHCl as the saline buffer helped to restore some of the sensitivity lost through using the divalent ions. Due to the altered sensitivity of the assay the standard curve was also made in saline rather than RIA buffer; i.e., RIA buffer was still used to dilute the antiserum and trace, but the sample volume of each tube consisted of 40µl Tris-buffered saline rather than RIA buffer. Finally, the values for the histograms were subjected to a running average of three to help reduce the effect of stray drops, and to clarify the overall pattern of release.

A comparison of the effects of the peptides on the isolated ventricle. The method of Payza (1987) was used to bioassay the peptides on ventricles isolated from aestivating Helix. In one case, the potencies of FLRFamide and FMRFamide were compared on ventricles from active animals. The taps were positioned as for the release experiments (Fig. 1). Each ventricle was perfused at a minimum rate of 600µl per minute (the rate varied between ventricles, but was constant for a given ventricle) with saline (80mM NaCl, 5mM KCl, 5mM $MgCl_2$, 7mM$CaCl_2$, 20mM HEPES, pH 7.5).

Some ventricles were recorded isotonically, others isometrically.

Doses (400µl) were injected via the 400µl injection loop. Two (occasionally three) peptides were tested on each ventricle, and each peptide pair or triplet was tested on a minimum of five ventricles. Roughly equipotent doses of each peptide were applied alternately 10 minutes apart. The transducer outputs were amplified and recorded on a J-J chart recorder. The percent increase in tension or amplitude was plotted against the log of the dose, and the potency ratio for the pair of drugs was estimated from the linear portion of the plots. Only one series of dose-response curves (i.e., one curve for each of the two or three peptides being compared) was obtained from each ventricle.

Materials

Helix aspersa were collected from Kingsbarns, Fife by Mr. J. Brown or shipped from California to St. Augustine, Florida by Dr. R. Koch or Night Bird Game and Fowl Co. Peptides were purchased from Peninsula Laboratories Inc., Cambridge Research Biochemicals or Sigma, or were synthesised by the University of Florida protein sequence core facility (SGYLAFPRMamide, NDPFLRFamide, NDPYLRFamide and SEPYLRFamide) or Dr. J. P. Riehm of the University of West Florida (SDPFLRFamide) (Ebberink, Price, van Loenhout, Doble, Riehm, Geraerts & Greenberg, 1987). Normal goat serum and goat-anti-rabbit conjugated FITC were purchased from Boehringer and Mannheim Biochemicals. The antisera S253 and Q2 were developed and kindly supplied by Dr. D. Price (Whitney Lab, University of Florida), and SCP_B by Dr. H. Morris (Imperial College, London). The remaining chemicals and supplies were purchased from Sigma or Fisher.

Results

Structure-activity relationships of S253 and Q2. Since these antisera were to be used in the RIAs to identify FMRFamide-related peptides in Helix, some measure of their specificity of binding was necessary. To satisfy this requirement, the structure-activity relationships (SARs) of S253 and Q2 were investigated. Exemplary binding curves for each of the two FMRFamide antisera are shown in figure 2; the curves were fitted by computer; i.e., the method of least squares was applied to the data points after log/logit transformation (a Hill plot).

A series of FMRFamide analogues and the two SCPs of Helix were tested in RIAs with S253 and Q2, and dose-response curves were prepared. The curves and IC_{50}s are shown in figure 3. Both antisera are most sensitive to the naturally occurring heptapeptides, and they are more sensitive to YGGFMRFamide, a synthetic heptapeptide analogue of FMRFamide, than to FMRFamide itself. Oxidation of the methionine in FMRFamide increases its IC_{50} by five to fifty fold. Removing the amide drastically reduces the affinity of the antisera to the peptide; indeed Q2 cannot detect even 50µl of 1mM FMRF and FLRF (50nmoles) constituting the entire sample volume of the assay. Neither antiserum is particularly sensitive to either of the SCPs, but both antisera recognise all the FMRFamide analogues endogenous to Helix. At the time that these binding curves were prepared, NDPYLRFamide and SEPYLRFamide had not yet been identified so these peptides were not included in the survey. Nevertheless, Q2, in particular, was used in the discovery of NDPYLRFamide and SEPYLRFamide (Price, Lesser, Lee, Doble and Greenberg, in prep.).

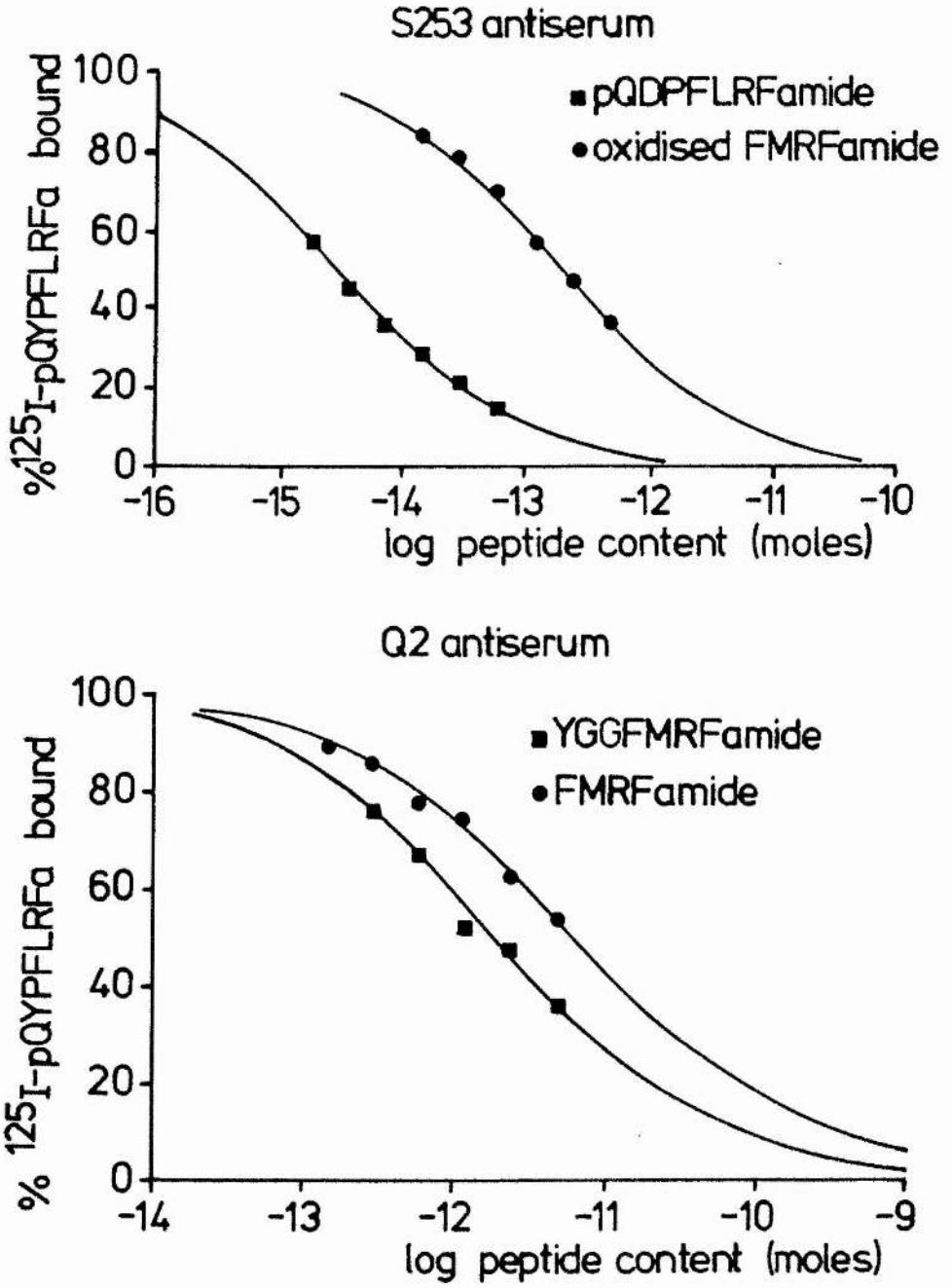
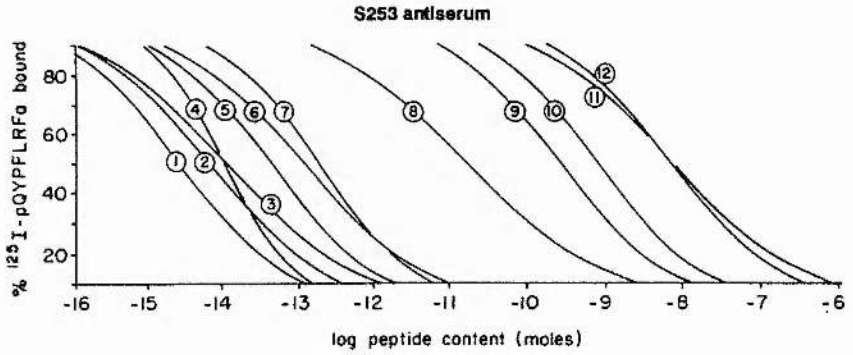


Figure 2. Examples of four of the binding curves shown in figure 3 and the computer calculated fit of the line. The curves were fitted to the points by computer according to the least squares fit to the log/logit transform equation calculated from the points. The log/logit transformation is also called a Hill plot; log(r/(1-r)) (Y-axis) is plotted against log[D] (X-axis) where r is the response as a proportion of the maximum response and [D] is the concentration of drug (peptide). The X-axis displays the peptide content of each assay vial. See figure 3 for a full explanation of the data.



S253 antiserum

	IC_{50} (moles)	Peptide
1	3×10^{-15}	pQDPFLRFa
2	7×10^{-15}	NDPFLRFa
3	1.2×10^{-14}	SDPFLRFa
6	1.27×10^{-13}	FLRFa
5	4.47×10^{-14}	FMRFa
7	2.03×10^{-13}	ox-FMRFa
4	1.2×10^{-14}	YGGFMRFa
8	1.7×10^{-11}	YGGFMRF
9	2.78×10^{-10}	MNYLAFPRMa
10	8.5×10^{-10}	SGYLAFPRMa
11	7.72×10^{-9}	FLRF
12	7.6×10^{-9}	FMRF

Q2 antiserum

	IC_{50} (moles)	Peptide
1	7.1×10^{-14}	pQDPFLRFa
2	1.73×10^{-13}	NDPFLRFa
3	2.64×10^{-13}	SDPFLRFa
4	3.74×10^{-13}	FLRFa
6	6.54×10^{-12}	FMRFa
7	3.04×10^{-10}	ox-FMRFa
5	1.9×10^{-12}	YGGFMRFa
9	4×10^{-8}	YGGFMRF
8	3.7×10^{-8}	SGYLAFPRMa
10	7.2×10^{-8}	MNYLAFPRMa

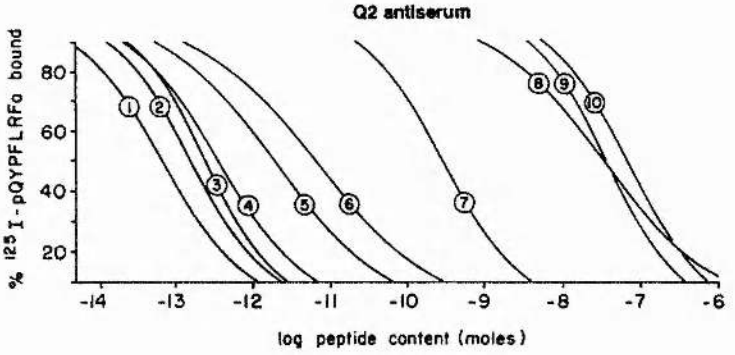


Figure 3. Binding curves to show the ability of the various peptides to inhibit the binding of the trace, ^{125}I-pQYPFLRFamide, to the two antisera S253 and Q2. The curves were fitted to the points by computer according to the least squares fit to the log/logit transform equation calculated from the points. For clarity, the points have not been shown. For both antisera the heptapeptides had a greater affinity for the antiserum than the corresponding tetrapeptide (pQDP-, NDP- and SDPFLRFamide had a greater affinity than FLRFamide and YGGFMRFamide had a greater affinity than FMRFamide). Oxidation of the methionine in FMRFamide reduced its affinity for both antisera. Removing the terminal amide from a peptide also reduced its affinity. The main difference between the two antisera is that S253 has a greater affinity for FMRFamide than for FLRFamide and Q2 has a greater affinity for FLRFamide than for FMRFamide. The peptides are listed in the same order for each antiserum which is not necessarily the order of potency.

The outstanding difference in the specificities of the two antisera is that S253 has a greater affinity for FMRFamide, and Q2 has a greater affinity for FLRFamide. (All the known endogenous FMRFamide analogues of Helix are recognised by Q2, and all but NDPYLRFamide and SEPYLRFamide are additionally recognised by S253.) Price (1983) and Lehman and Price (1987) also prepared some competition curves for the S253 antiserum, but used iodinated YGGFMRFamide for the trace instead of iodinated pQYPFLRFamide. Not surprisingly, the assay was more sensitive to FMRFamide and YGGFMRFamide. The advantage of using pQYPFLRFamide as the trace is that, by binding less well to the S253 antiserum, it increases the sensitivity of the assay. Moreover, it can also be used with the Q2 antiserum, a practical matter.

Differential distribution of the peptides in cardiac and nervous tissue. Acetone extracts of selected Helix tissues were separated by HPLC and analysed by RIA. An illustrative HPLC separation of standard synthetic peptides is shown in figure 4: aliquots containing 1nmole of the indicated peptides were injected; the UV absorbance (210 and 280nm) of the column effluent is displayed. The circumoesophageal ring (brain) contains all seven FMRFamide analogues and both SCPs (Fig. 5). Moreover, all nine peptides have each been isolated previously from the circumoesophageal ganglia and subsequently analysed for their animo acid content if not actually sequenced (see Introduction for references). Analysis of the visceral and anal nerves combined shows that they, too, contain all nine peptides (Fig. 6) as does the anterior aorta (Fig. 7). The SCPs and both tetrapeptide amides are present in the heart, but the

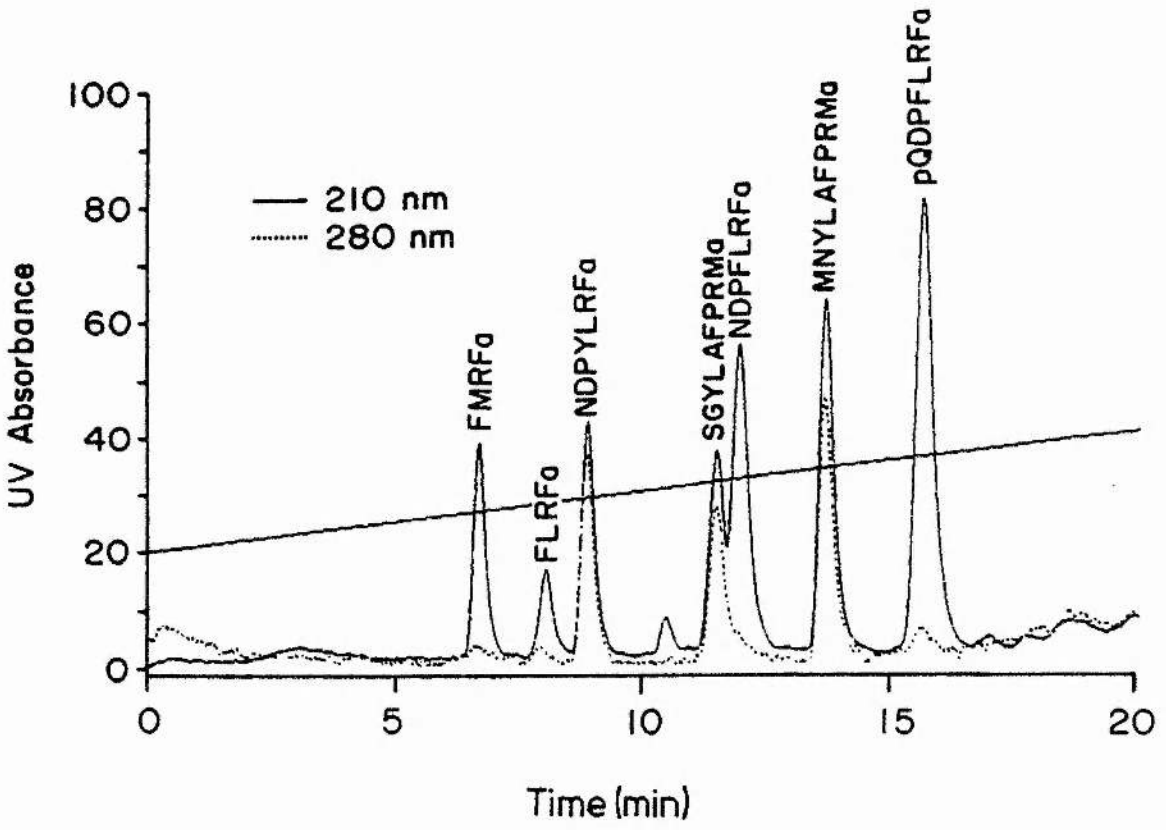


Figure 4. HPLC traces of the UV absorbance (210 and 280nm) of the effluent of the RP-300 column to show the approximate elution times and order of elution of the injected standards (a mixture of 1 nmole of each standard was injected onto the column through the injector). This trace was kindly supplied by D. Price.

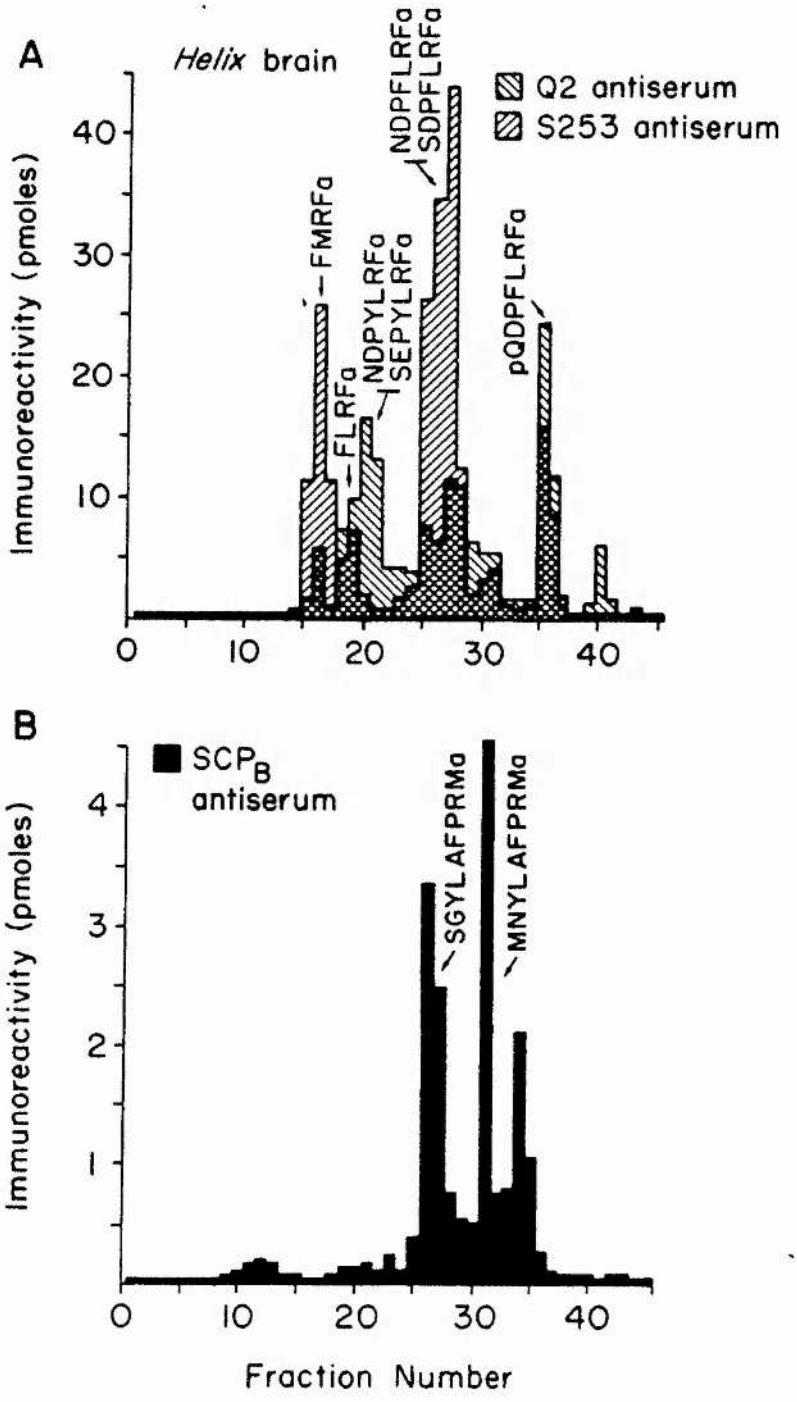


Figure 5. RIA analysis of HPLC fractions of acetone extracts of the circumoesophageal ring. A. The peaks identified with S253 antiserum are superimposed upon those identified with Q2. B. The same fractions analysed with SCP_B antiserum. All nine peptides are present.

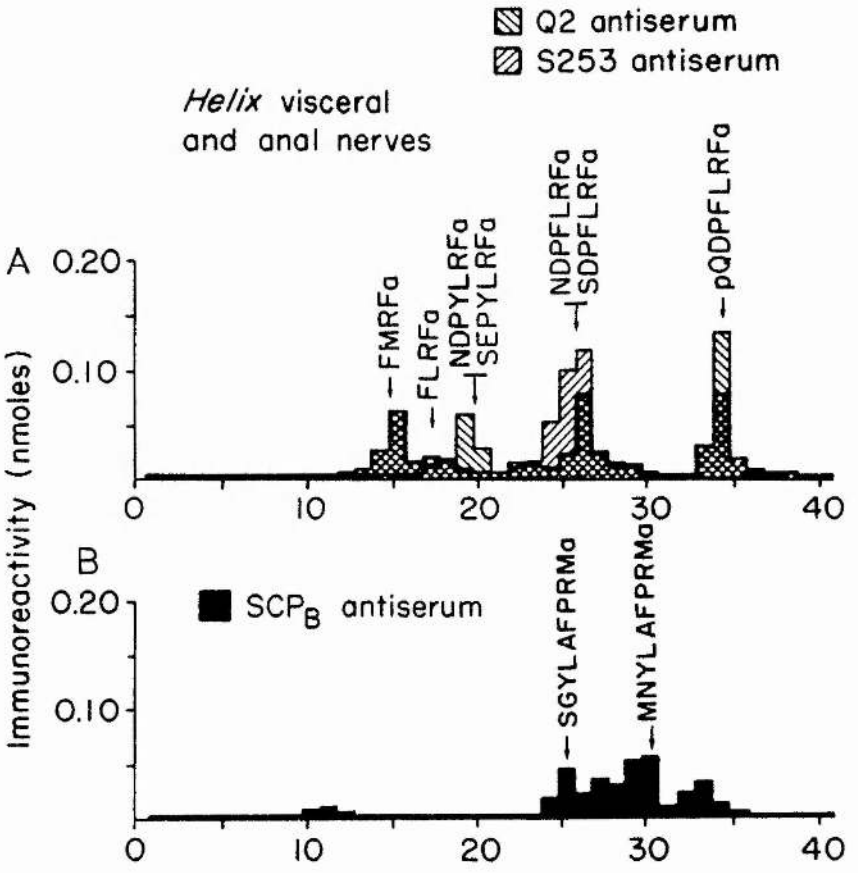


Figure 6. RIA analysis of HPLC fractions of acetone extracts of the visceral and anal nerve trunks. A. The peaks identified with S253 antiserum are superimposed upon those identified with Q2. B. The same fractions analysed with SCP_B antiserum. All nine peptides are present.

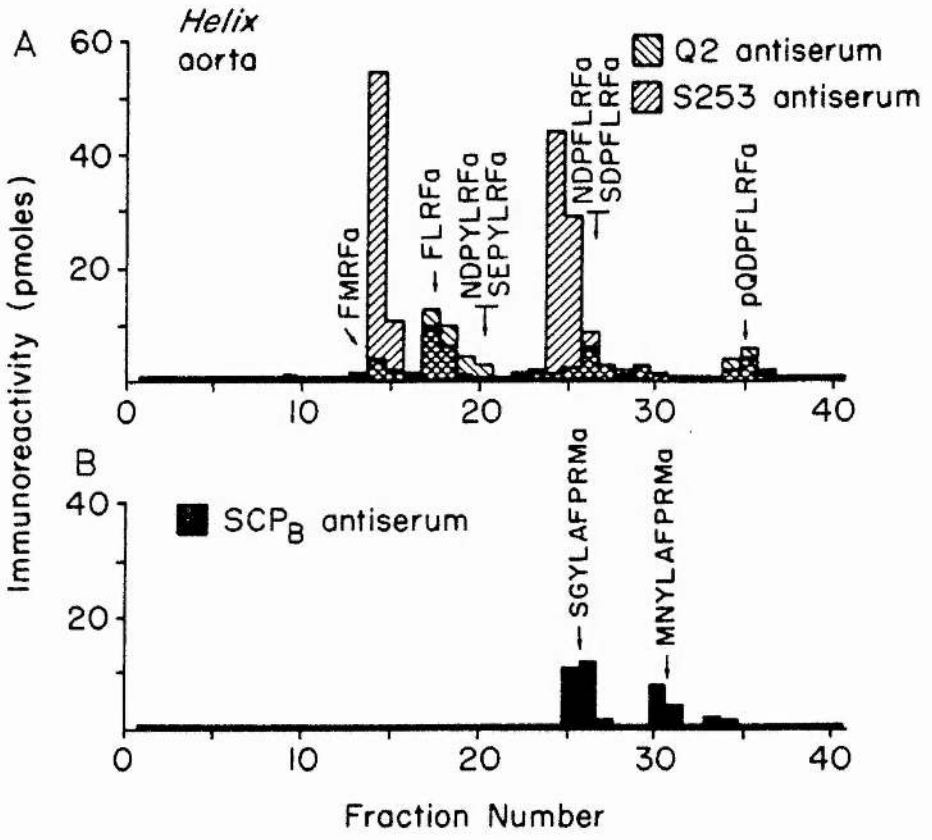


Figure 7. RIA analysis of HPLC fractions of acetone extracts of the anterior aorta. A. The peaks identified with S253 are superimposed upon those identified with Q2. B. The same fractions analysed with SCP_B antiserum. All nine peptides appear to be present.

heptapeptides are notable for their absence from this tissue (Fig. 8). As the third large peak recognised by S253 in the heart is not recognised by Q2, it can not be one of the identified FMRFamide analogues; i.e., all of them (except FMRFamide) contain an antepenultimate leucine residue).

Distribution of immunoreactivty within the heart and nervous system. Immunohistochemistry of the heart with S253 revealed a diffuse network of nerves evenly distributed throughout the heart (Fig. 9). Some varicosities can also be seen. The staining appears to follow the trabeculae, indicating that the fibres are on, or just below, the luminal surface of the heart, rather than deeply embedded within the tissue.

Positive staining within the suboesophageal ring was seen in two clusters of cell bodies; one in each of the two parietal ganglia (Fig. 10). A positively stained bundle of processes can also be seen in the visceral nerve as it leaves the ganglion. Other workers have stained sections of the central ganglia with FMRFamide antisera (Marchand, Wijdenes & Schot, 1982; Cottrell, Davies, Turner & Oates, 1988).

The main advantage of immunohistochemistry on wholemounts as compared with sections is that the orientation of the intact tissue is obvious, and so the different regions of the tissue are easily identified. However one possible drawback of wholemounts can be seen in the photograph of the atrial-ventricular junction (Fig. 9, middle). The atrial wall is much thinner than the ventricular wall, so the background fluorescence is much higher in the ventricle and the weaker staining is not so visible. The suboesophageal ganglia are thicker still (Fig. 10) so only the

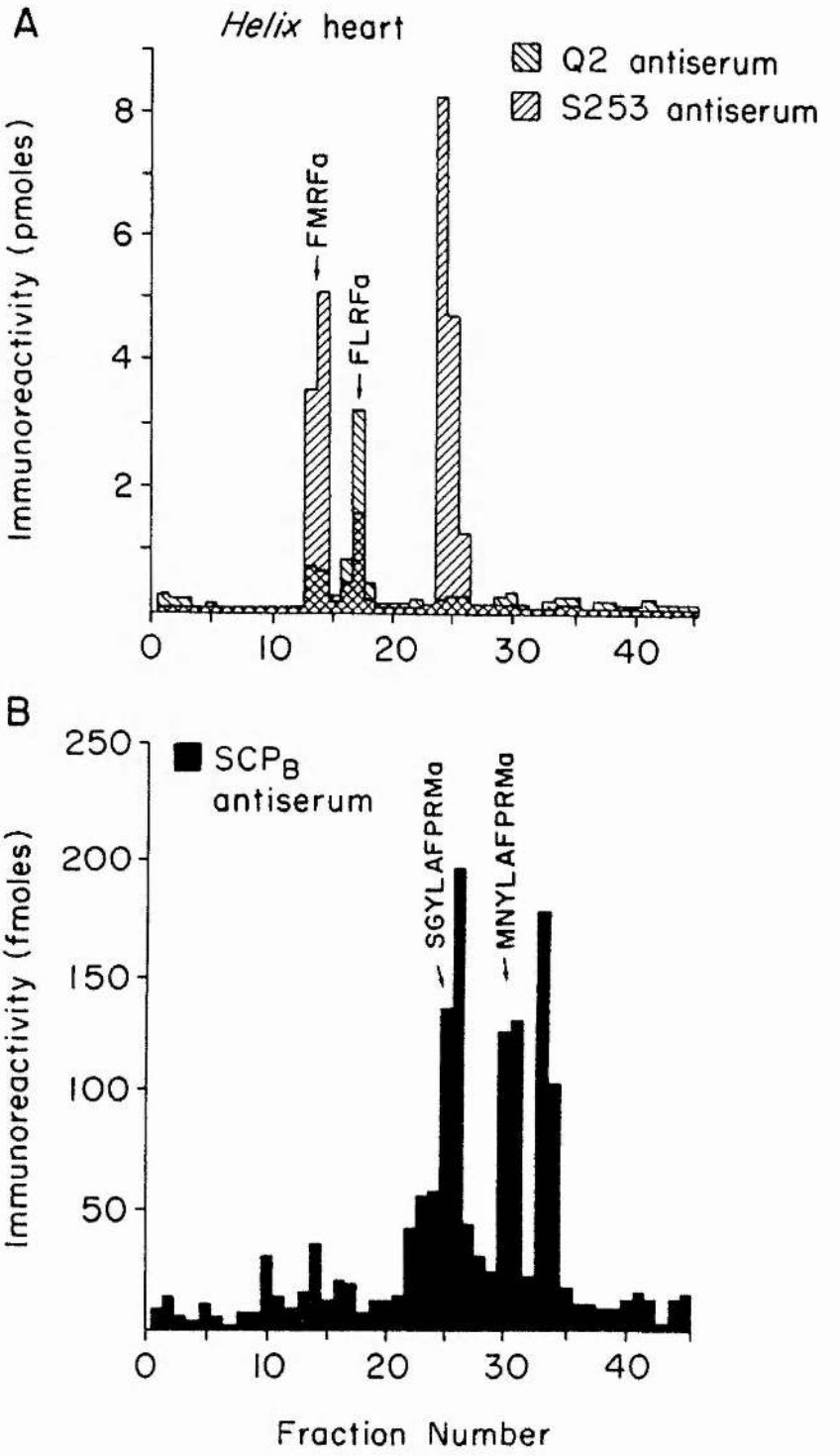
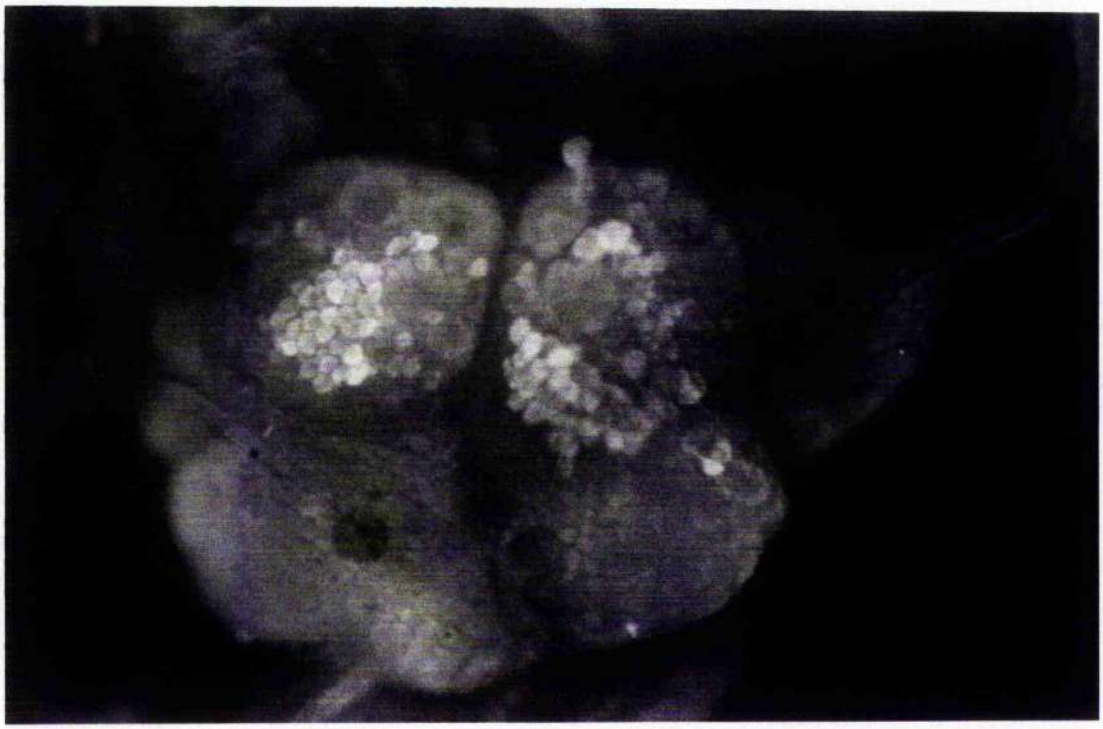


Figure 8. RIA analysis of HPLC fractions of acetone extracts of the heart. A. The peaks identified with S253 antiserum are superimposed upon those identified with Q2. B. The same fractions analysed with SCP_B antiserum. Note that the heptapeptides are missing.

Figure 9. Immunohistochemistry of the heart with S253 antiserum (wholemount). Atrium (top). A-V junction (middle). Ventricle (bottom). Magnification x 100.

Figure 10. Immunohistochemistry of the suboesophageal ganglia with S253 antiserum (wholemount). Magnification x 60.



very bright staining is obvious. Thus the two groups of stained neuronal soma stand out against the background, but the bundle of nerve fibres in the visceral nerve are less easily noticed; understandably, the single axons leaving each soma are even less visible.

One method for overcoming the poor visibility is to clear the tissue with xylene or methylsalicylate, a procedure that works well with sections. Attempts to clear these wholemounts have, however, been unsuccessful. The problem is that the tissues must be pinned out before dehydration begins and then unpinned before mounting (when they are very fragile), and this requires a microscope and lighting. Unfortunately, even a low intensity, non-fluorescent light causes the fluorescence to fade markedly, thus ruining the preparation.

Finally, S253 does not seem to bind well to the two tyrosine analogues (NDPYLRFamide and SEPYLRFamide), so unless these two analogues always coexist with one of the other peptides, their locations will not be identified by this antiserum.

Calcium-dependent release of the FMRFamide peptides. The demonstration of the calcium-dependent release of FMRFamide peptides from the heart was straightforward (Fig. 11A). A tenfold increase in the potassium concentration of the perfusion saline had no effect when the calcium content was nominal zero. When the calcium level was restored, FMRFamide peptides were released when the potassium level was raised (Fig. 11A).

A calcium-dependent release of FMRFamide peptides from the brain was more difficult to show (Figs. 11B, 12). Perfusing the brain with saline containing nominal zero calcium appeared to

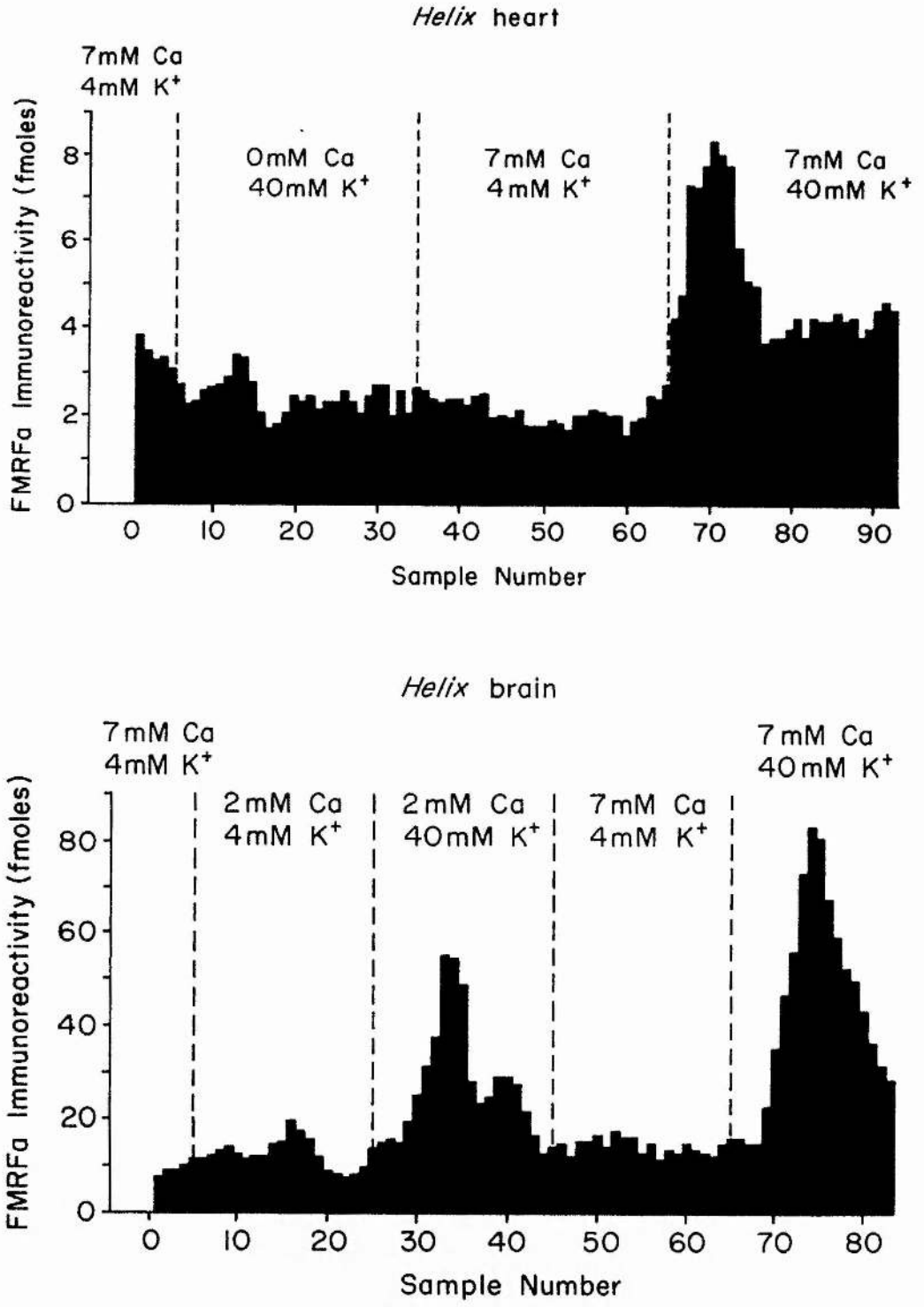


Figure 11. Demonstration of the release of FMRFamide peptides from the heart (top), and the circumoesophageal ring (bottom). The calcium and potassium content of the saline perfusing the tissue at the time of collection of each drop is indicated along the top of each figure. Each bar of the histogram represents the FMRFamide immunoreactivity of a 40µl drop.

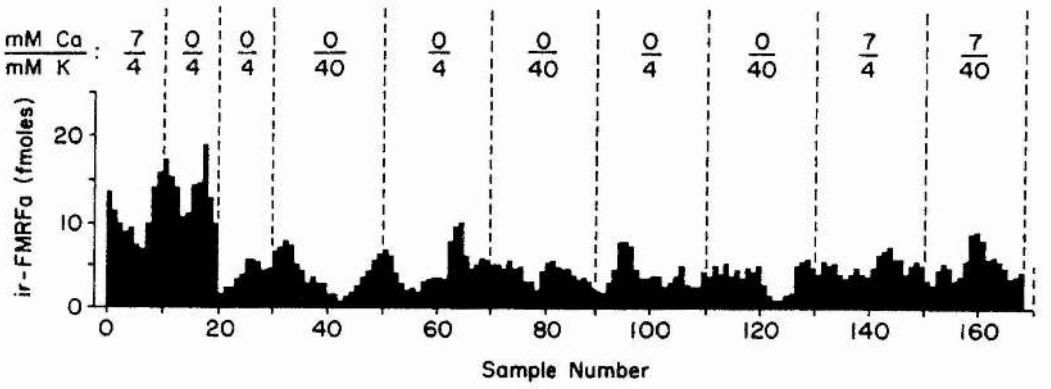


Figure 12. The effect of nominally zero calcium saline on the basal release of the circumoesophageal ring. There is a thirty minute gap between fractions 19 and 20, during which time the basal release has fallen substantially. The basal level does not return to its original value even when the ring is perfused with saline containing the normal calcium concentration.

cause irreversible damage. In the presence of normal potassium levels, zero calcium did not stimulate release; rather, it depressed the basal level permanently (Fig. 12). However, preincubation in 2mM calcium, in the presence of normal potassium, was tolerated by the brain (Fig. 11B). When the potassium level was raised tenfold, some release of the peptides was observed. Replacing this modified saline with the original saline restored the calcium levels and presumably allowed the tissue to repolarise. When the potassium level was raised in the presence of normal calcium levels the subsequent release was of greater magnitude and duration (Fig. 11B).

Comparison of the Actions of the Peptides on the Isolated Cardiac Ventricle

FMRFamide analogues. The effects of the FMRFamide analogues on ventricles isolated from aestivating animals were qualitatively similar to each other (Figs. 13, 14, 15). Usually, the beat amplitude rather than the frequency was increased; but effects on frequency could sometimes be seen (Fig. 16B), as could increases (Fig. 13E) and decreases (Fig. 15B) in tone.

The peptides have different potencies; the potency ratio between the least and the most potent being one hundredfold (Fig. 13C). The five heptapeptide analogues were equipotent (Figs. 13D, 14). Increasing the peptide length increased potency. The largest potency increase due to the addition of a single residue (a factor of eight) accompanied the extension of FLRFamide to PFLRFamide (Figs. 15A, B); the smallest such increase (a factor of only two) accompanied the subsequent extension of PFLRFamide to DPFLRFamide (Figs. 15C, D). The apparent increase in potency

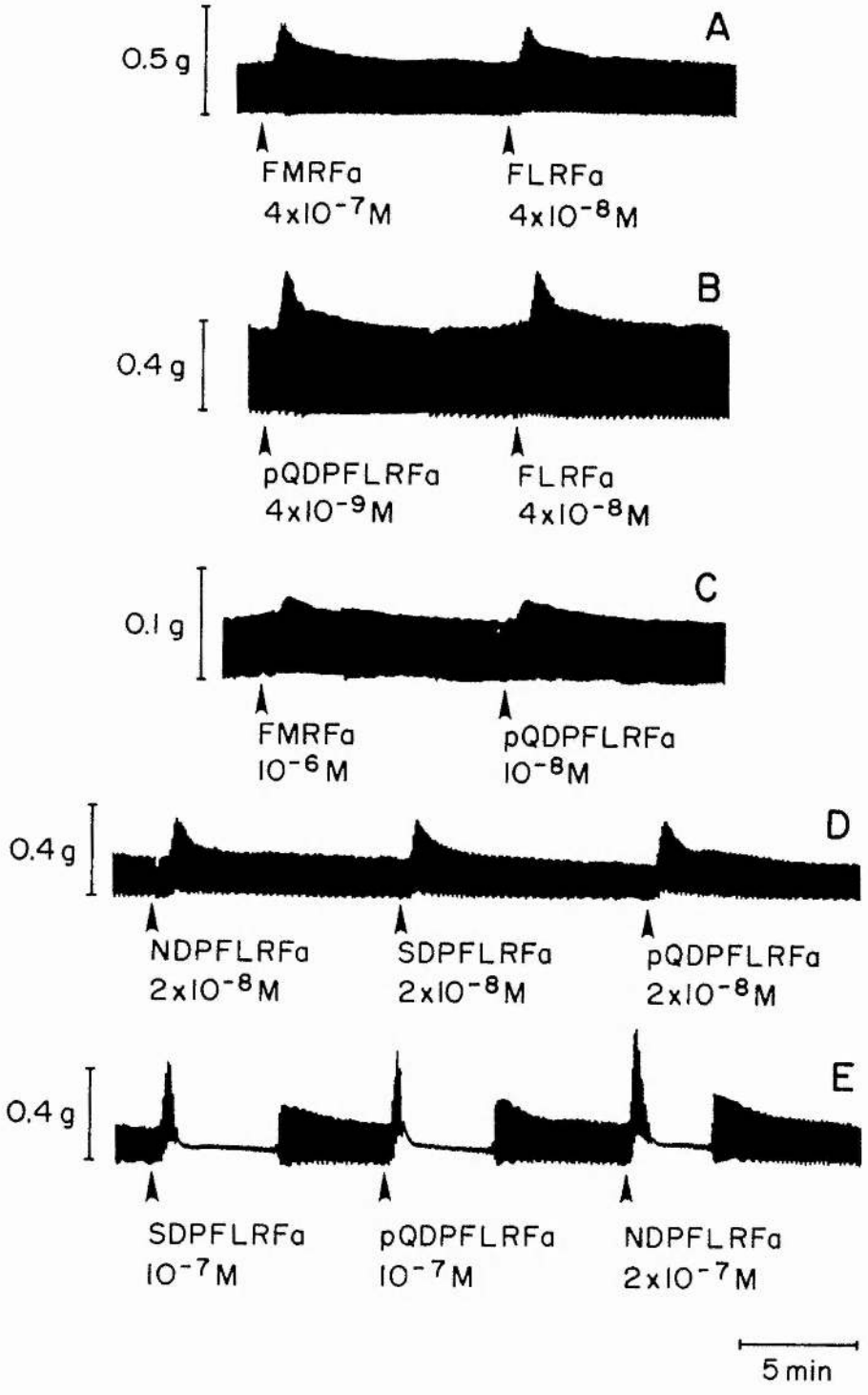


Figure 13. Pairs (A-C) and triplets (D-E) of approximately equipotent doses of the FMRFamide peptides recorded isometrically. 400µl of the peptide at the concentration indicated below each dose was injected into the sample loop at the arrows. The five sets of traces are from five different ventricles.

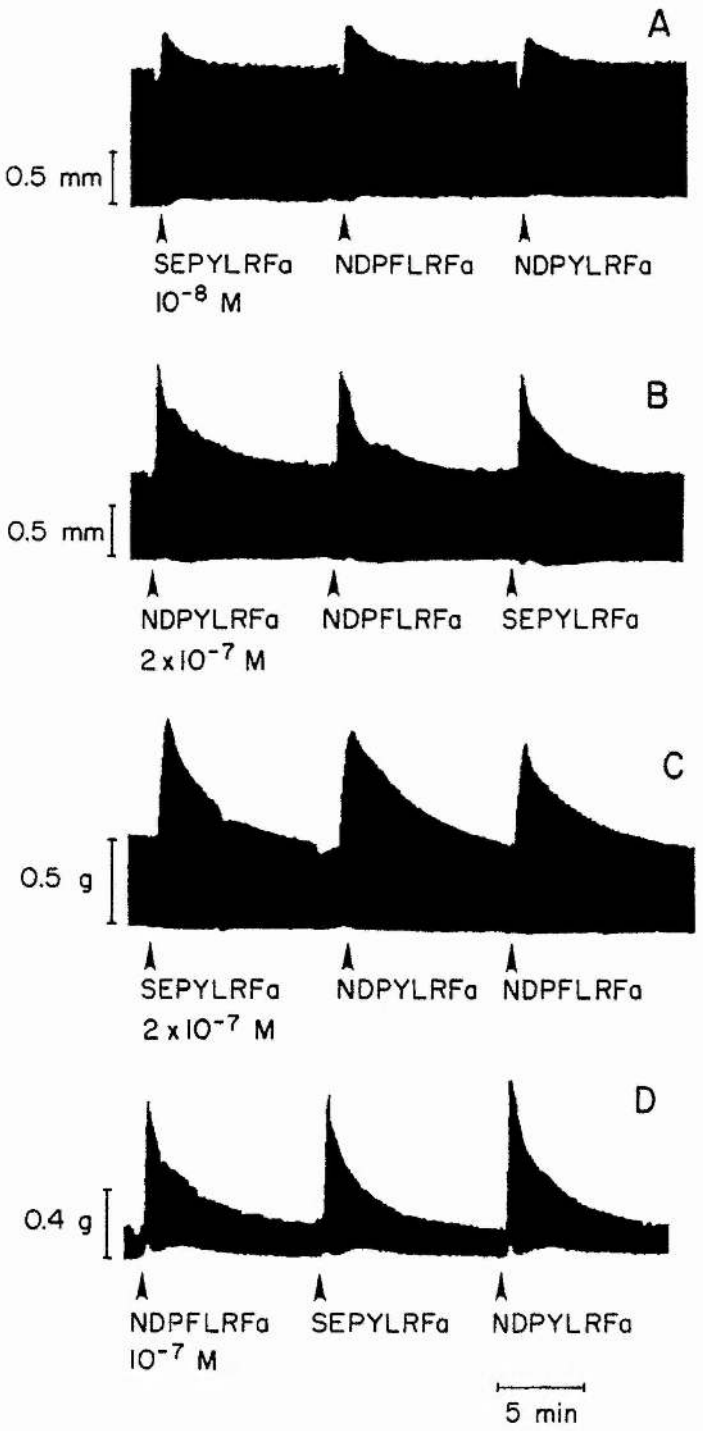


Figure 14. Triplets of approximately equipotent doses of the tyrosine analogues and NDPFLRFamide recorded isotonically (A,B) and isometrically (C,D). 400µl of the peptide at the concentration indicated below each dose was injected into the sample loop at the arrows. The four traces are from four different ventricles. Although the peptides did not necessarily seem equipotent on a particular ventricle, the more potent peptide varied with each ventricle. Thus NDPYLRFamide is the least potent peptide in record A but the most potent in record D so that, overall, the three heptapeptides are equipotent.

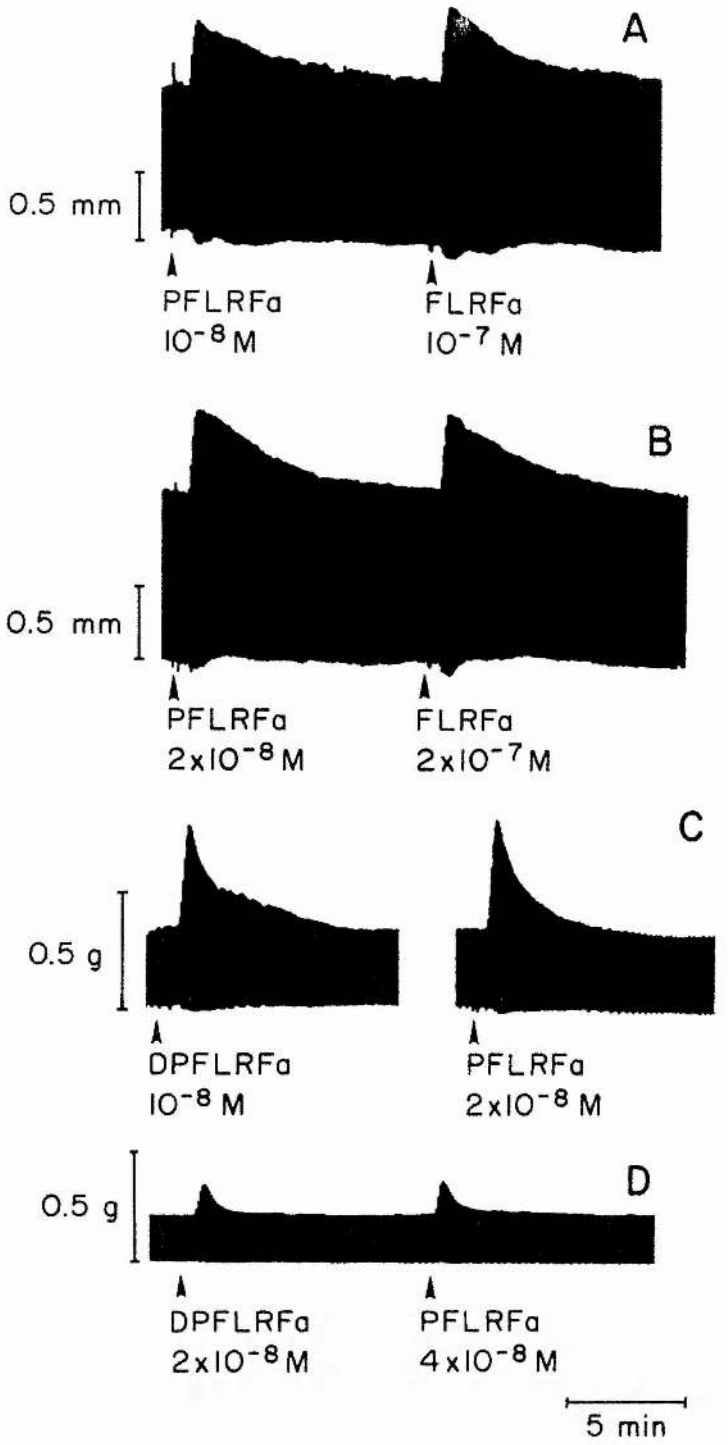


Figure 15. Pairs of approximately equipotent doses of FLRFamide and the synthetic analogues PFLRFamide and DPFLRFamide recorded isotonically (A,B) and isometrically (C,D). 400µl of the peptide at the concentration indicated below each dose was injected into the sample loop at the arrows. The break in trace C indicates that the doses were not consecutive, though they are shown in the order of application. Records A and B are from one ventricle, C and D are from two others.

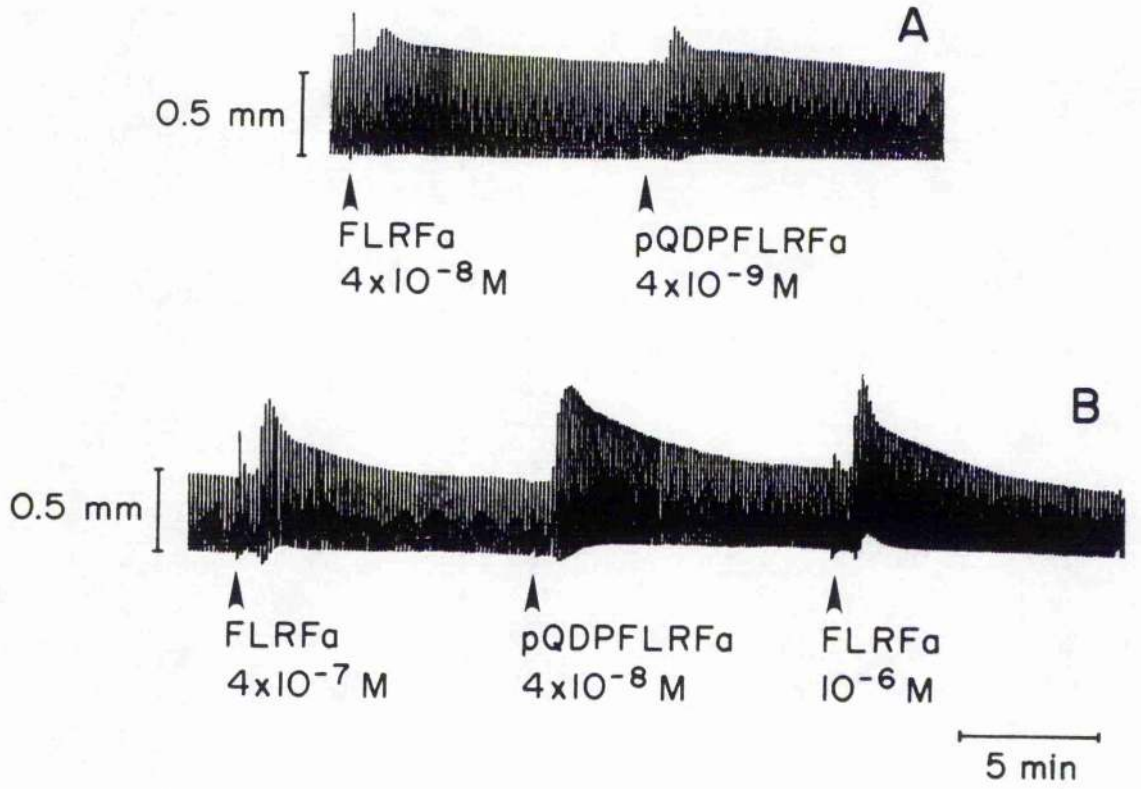


Figure 16. Two sets of responses from the same ventricle to FLRFamide and pQDPFLRFamide. They are recorded isotonically. The ventricle was beating at a sufficiently slow rate for the individual beats to be distinct. There is a break between the two traces. 400µl of the peptide at the concentration indicated below each dose was injected into the sample loop at the arrow. Although there is no obvious chronotropic effect visible in trace A, there is a positive chronotropic effect in the response to 4×10^{-8}M pQDPFLRFamide in trace B.

may not be due solely to the protection against enzyme degradation afforded by length increases; i.e., although pQDP-, NDP- and SDPFLRFamide are all equipotent, pQDPFLRFamide is N-terminally blocked and is therefore completely resistant to degradation by amino peptidases. Additionally, these comparisons were made on saline-perfused (i.e., no blood) isolated ventricles where the action of degrading enzymes should be minimal if present at all.

The FLRFamide heptapeptides could inhibit or even arrest the ventricle (Fig. 13E). The tendency of a particular peptide to be cardioinhibitory was partly determined by the peptide with which it was paired in testing. FMRFamide never arrested the ventricle, and FLRFamide never caused arrest when tested with FMRFamide. But FLRFamide did arrest two of six ventricles when paired with pQDPFLRFamide. pQDPFLRFamide arrested only one of six ventricles when paired with FLRFamide, but arrested four of six ventricles when paired with NDPFLRFamide and SDPFLRFamide (Fig. 13E). The significance of these observations is unknown at present; the possible interdependence of the peptide actions requires further experiments.

SCPs. The responses of the ventricles to the SCPs were qualitatively and quantitatively distinguishable from those to the FMRFamide analogues (Fig. 17). The two SCPs elicited very similar responses, though SGYLAFPRMamide was consistently about three times more potent than MNYLAFPRMamide. As these two peptides were standardised to each other by HPLC (D. Price, personal communication) this difference, though small, is probably reliable. At low doses, the SCPs increased the beat amplitude and often decreased tone. Chronotropic effects were not observed.

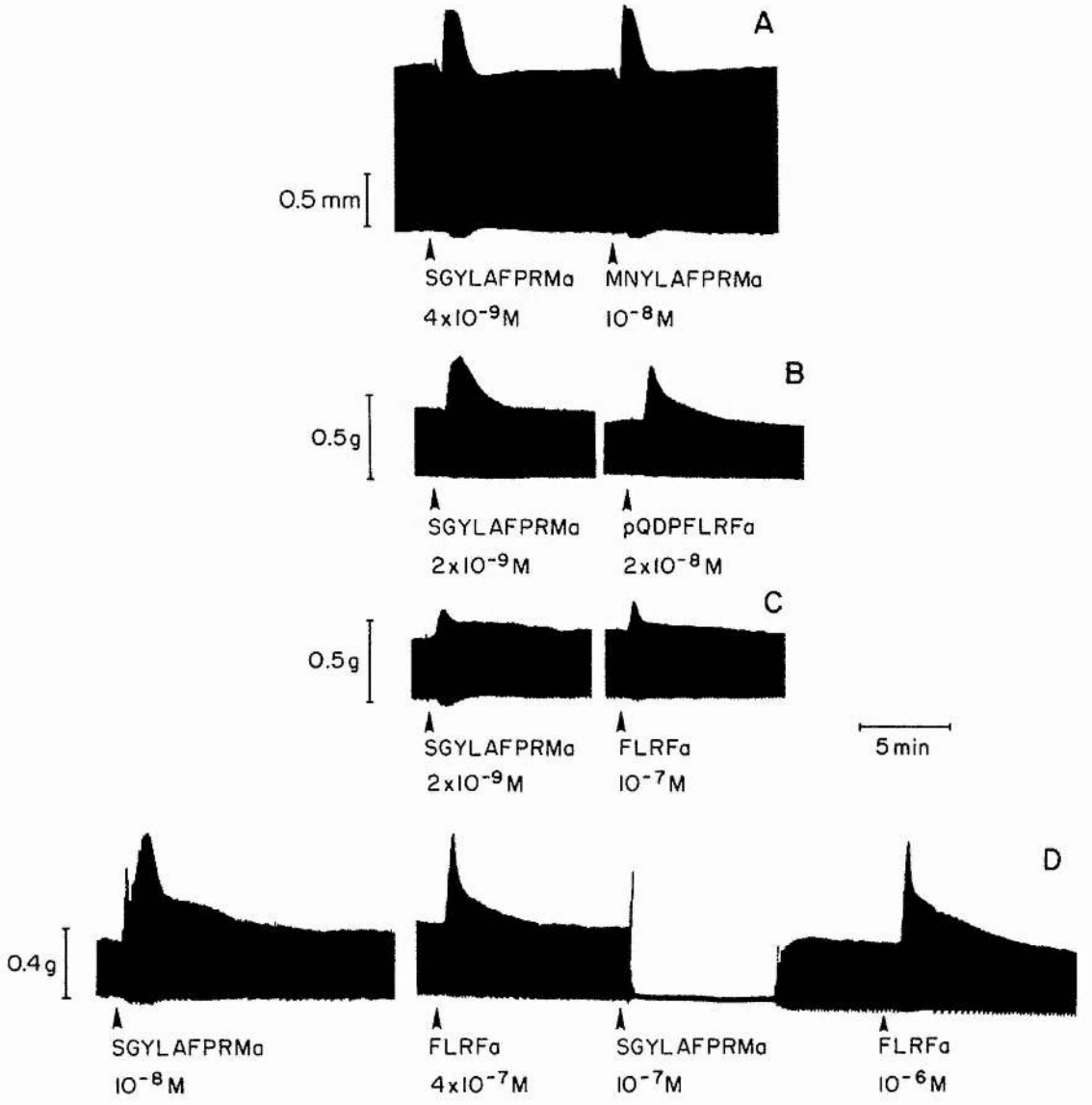


Figure 17. Pairs (A-C) of approximately equipotent doses of the SCP and FMRFamide peptides recorded isotonically (A) and isometrically (B-D). 400µl of the peptide at the concentration indicated below each dose was injected into the sample loop at the arrows. The break in the trace indicates that the doses were not consecutive, though they are shown in the order of application. D. The 10^{-8}M dose of SGYLAFPRMamide and 4×10^{-7}M dose of FLRFamide were approximately equipotent. The 10^{-7}M dose of SGYLAFPRMamide arrested the ventricle but did not seem to affect the subsequent FLRFamide response. Each of the four records is from a different ventricle.

Comparison of the effects of FMRFamide analogues and SCPs. SGYLAFPRMamide was also compared with FLRFamide and pQDPFLRFamide (Figs. 17B, C); these records illustrate the dissimilar effects of the SCPs and the FMRFamide analogues. The SCP responses were more sustained at their peak (i.e., they were more rounded at the top) than those of the FMRFamide analogues (more pointed responses) (Figs. 17B, C). They may also show split peaks at higher doses (Fig. 17D). The SCPs often reduced diastolic tone, whether the recording was isotonic or isometric (Figs. 17A, C). Like the heptapeptides, they could inhibit and arrest the ventricle (Fig. 17D). The eight minute arrest caused by SGYLAFPRMamide in figure 17D had no obvious effect on the subsequent FLRFamide response, so the SCPs may act through a different mechanism on the ventricle than that of the FMRFamide analogues.

Occasionally some equipotent pairs of responses displayed more obvious qualitative differences. Some examples are shown in figure 18. The height of the sharp initial peak was measured in the determination of the response of the ventricle to these particular doses of peptide. However, secondary shoulders could sometimes be seen following these initial peaks. These shoulders were more prominent in one member of the pair than the other.

Figure 19 summarises the potency ratios between the different peptides on the isolated ventricle from aestivating Helix.

Comparison of the relative potencies of FMRFamide and FLRFamide on ventricles from aestivating and active animals. The responses of four ventricles to FMRFamide and FLRFamide can be seen in figure 20: A-C were ventricles from active animals; D

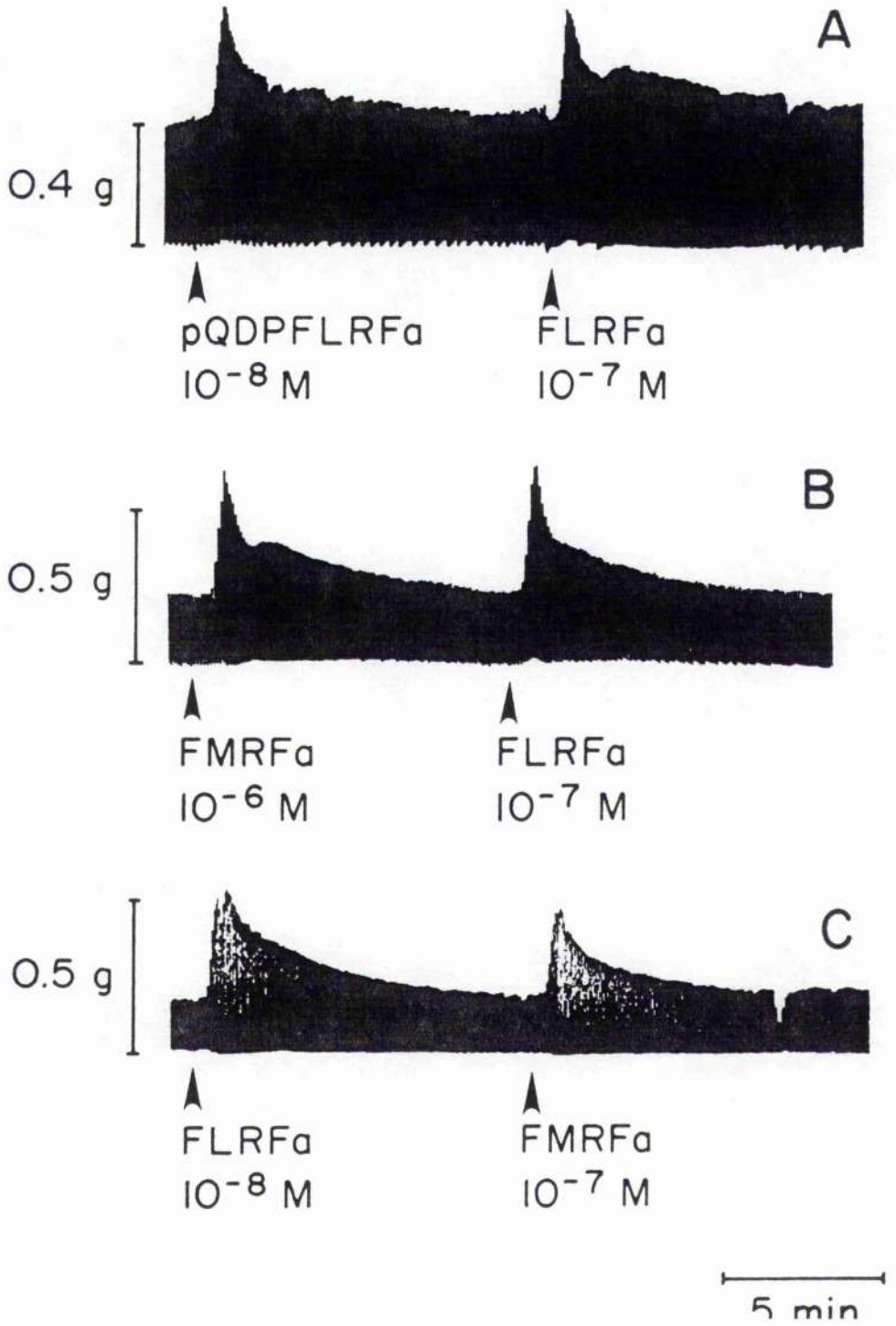


Figure 18. Pairs of approximately equipotent doses of FMRFamide peptides applied to three isolated ventricles from aestivating Helix. 400µl of the peptide at the concentration indicated below each dose was injected into the sample loop at the arrows. Small qualitative differences between the members of each pair can be seen (mainly whether or not a shoulder is obvious in the response).

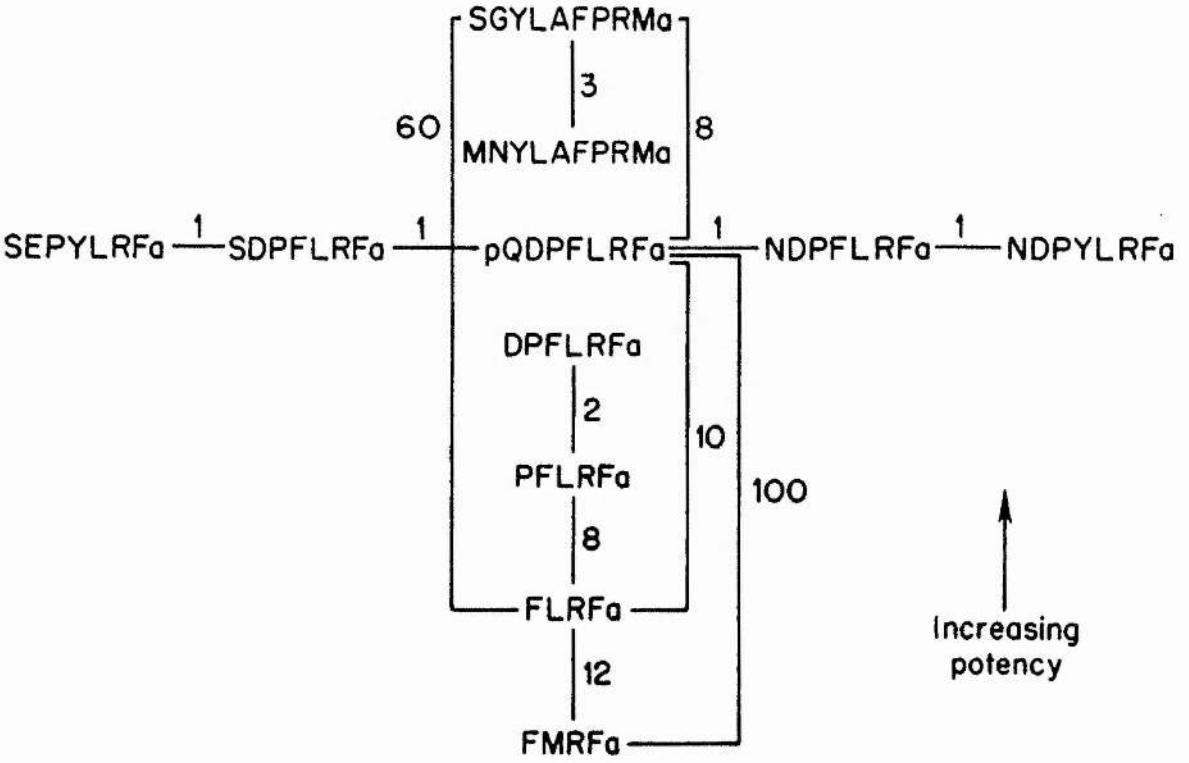


Figure 19. The relative potencies of the peptides on the isolated ventricle from aestivating Helix aspersa. The order of potency decreases from top to bottom.

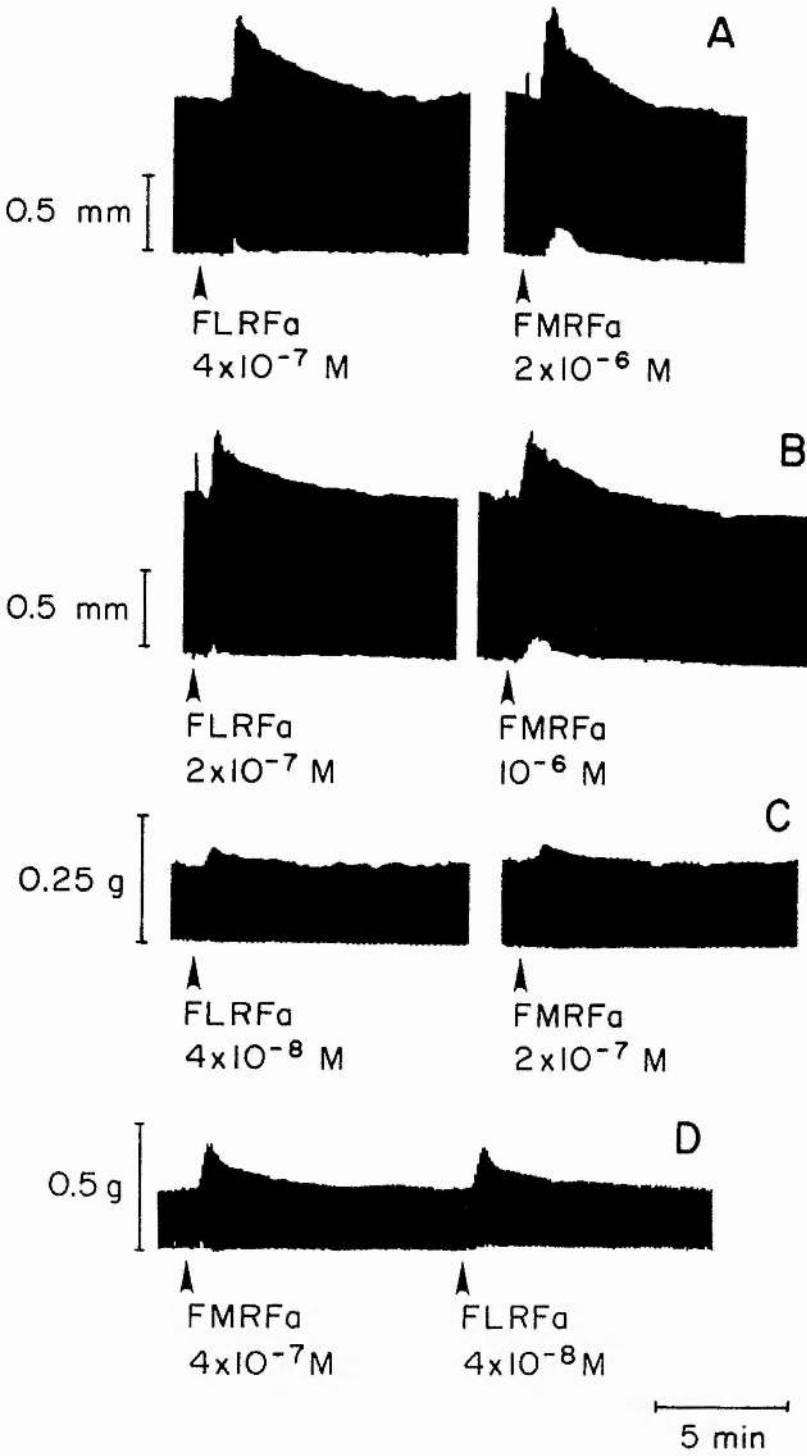


Figure 20. Pairs of approximately equipotent doses of FLRFamide and FMRFamide applied to isolated ventricles from active (A-C) and aestivating (D) Helix. 400µl of the peptide at the concentration indicated below each dose was injected into the sample loop at the arrows. Record D is the same as that in figure 13A. Records A and B are from one ventricle, C and D are from two other ventricles.

was from an aestivating animal and is the same record shown in figure 13A. A comparison of parts C and D, both from isometric recordings, reveals no obvious qualitative difference, either between the two peptides, or between the two states of activity. Likewise parts A and B are similar; the increase in tone observed was probably more a function of the isotonic recording than the state of the animal. Table 3 lists the potency ratios (mean +/- the standard error of the mean) of the peptide pairs tested; the more potent analogue is listed first.

Peptide pair	N	mean +/- s.e.m.
AESTIVATING		
SGYLAFPRMamide/FLRFamide	5	59.806 +/- 13.188
SGYLAFPRMamide/pQDPFLRFamide	5	7.948 +/- 3.614
SGYLAFPRMamide/MNYLAFPRMamide	5	2.798 +/- 0.169
pQDPFLRFamide/FMRFamide	3	97.72 +/- 25.63
pQDPFLRFamide/FLRFamide	6	9.288 +/- 1.236
DPFLRFamide/PFLRFamide	5	1.853 +/- 0.236
PFLRFamide/FLRFamide	8	8.379 +/- 1.925
FLRFamide/FMRFamide	5	11.694 +/- 2.63
ACTIVE		
FLRFamide/FMRFamide	5	4.89 +/- 0.623

Table 3. The mean +/- standard error of the mean (s.e.m.) for each peptide pair tested on isolated Helix ventricles. The first eight pairs were tested on ventricles from aestivating animals, the final pair was tested on ventricles from active animals. N is the number of ventricles considered in the calculation of the mean and standard error for each pair.

The potency ratios for FLRFamide and FMRFamide on ventricles removed from aestivating animals was 11.694 +/- 2.63, while that for ventricles removed from active animals was 4.89 +/- 0.623. Analysis of these differences with the Students T-test showed

that this difference is significant at $p<0.001$; i.e., the probability that both sets of data are from the same population is less than one in one thousand.

Dose-response curves. A selection of dose-response curves are shown in figure 21. The straight sections of the curves are approximately parallel for each set of responses. Each peptide was added over a set concentration range which had been previously determined to include the dose that gave a maximal or near-maximal response, or (in the case of the tetrapeptides) a rather high concentration (4×10^{-6}M) had been reached. Comparison of two or more curves for the same peptide reveals the variety of ventricular response. For example, SGYLAFPRMamide elicited a "classical" curve in figure 21B and F, evoked reduced excitation in figure 21C and arrested the ventricle in figure 21A. The maximal response was not easy to define in the presence of inhibition and cardiac arrest, and the maximal responses were often not the same for both members of a particular pair of peptides (Fig. 21A). SGYLAFPRMamide arrested the ventricle in figure 21A so its curve is incomplete, and pQDPFLRFamide arrested the ventricle in figure 21H so both curves are incomplete. The remaining curves are complete. Thus it seemed that the most simple and objective criterion to compare between peptides was the equipotent dose using the linear section of the curves.

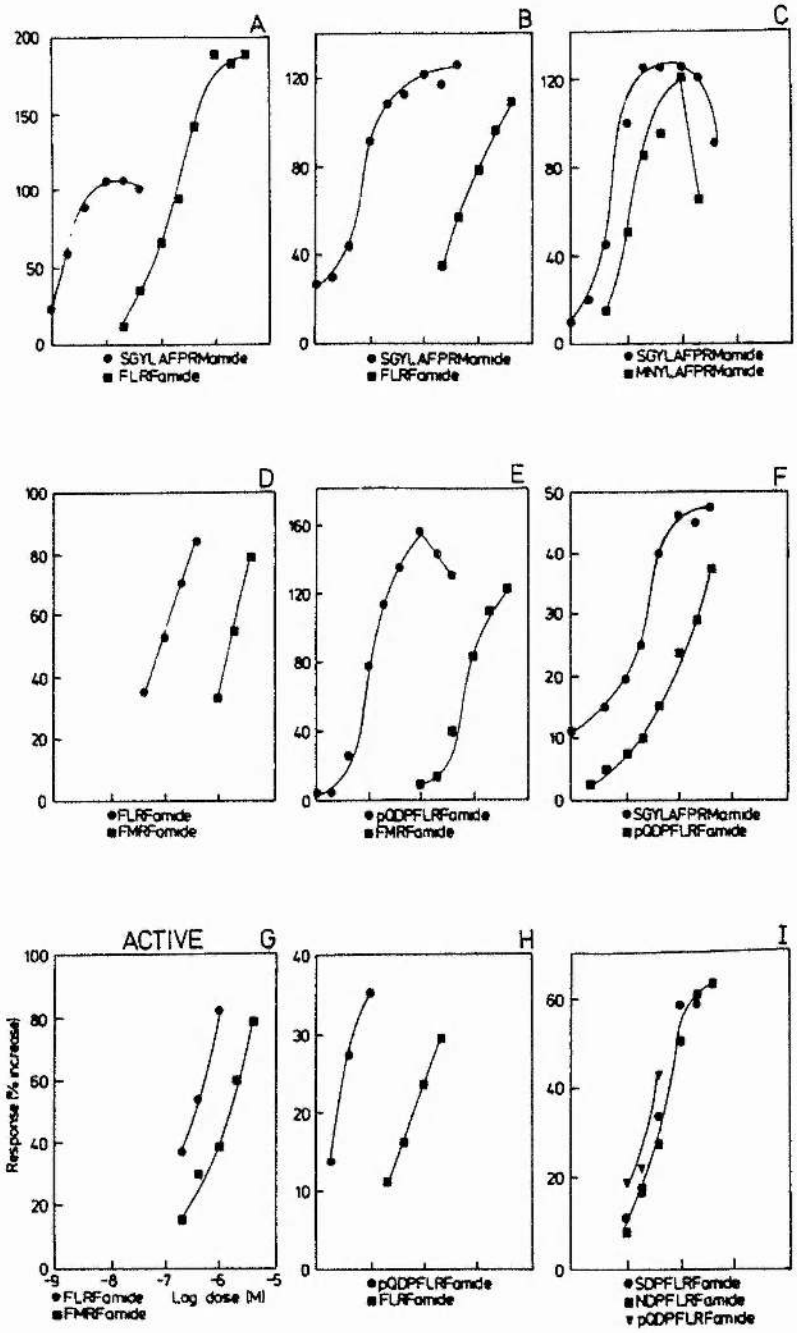


Figure 21. Dose-response curves for the endogenous peptides on ventricles from aestivating Helix (A-F, H, I). All the curves for a set are from the same ventricle though each set is from a different ventricle. Set G is from a ventricle from an active animal. The X-axis is constant but the Y-axis (% increase in beat strength or amplitude) varies between sets. SGYLAFPRMamide arrested the ventricle in set A so its curve is incomplete. pQDPFLRFamide arrested the ventricle in set H so both curves are incomplete. The remaining curves are complete and show that while the linear portions of the curves are parallel, the maximal response was not always evoked, though it was exceeded in some curves where higher doses elicit sub-maximal responses.

Discussion

Summary of the evidence. The nine endogenous peptides of Helix are all active on the isolated Helix ventricle, but they are not equipotent. The tetrapeptides are the least potent; the SCP nonapeptides are the most potent; and the heptapeptides, equipotent with each other, are of intermediate potency. Immunohistochemical staining with FMRFamide antisera reveals a diffuse nerve network in the Helix heart, two clusters of cell bodies in the suboesophageal ganglia, and nerve processes in the visceral nerve. Analysis of these tissues by HPLC and subsequent RIA shows that all of the peptides are present in the central nervous tissues, but that the heptapeptides are missing from the heart. The mean concentration of pQDPFLRFamide in the blood of Helix aspersa is 3-7nM (Price, Cottrell, Doble, Greenberg, Jorenby, Lehman & Riehm, 1985). The calcium-dependent release of FMRFamide-immunoreactive material from both the heart and the circumoesophageal ring suggests that these peptides may be released from these two organs under physiological conditions.

Effects of FMRFamide and possible presence of an analogue in the anterior aorta. Positive staining with a FMRFamide antiserum has been observed in the outer, longitudinal layer of the anterior aorta of Helix aspersa, but this layer is unresponsive to synthetic FMRFamide in organ bath experiments. In contrast, the inner, circular muscle contracts in the presence of FMRFamide, but does not exhibit positive staining with FMRFamide antiserum (Griffond, Boer & Wijdenes, 1986). The authors propose that the active substance is carried to the outer muscle layer in nerves, whence it is released in a non-synaptic

manner, to diffuse to the circular layer where it exerts its effect. The actual substance (if, indeed there is only one) has not been identified, and it could be any of the endogenous FMRFamide analogues: all seven have been extracted from the anterior aorta in acetone, and all have been identified by HPLC and RIA analysis; but their actions on the aorta have not yet been compared. Thus, in addition to strengthening the heartbeat, the FMRFamide peptides could influence the regulation of blood flow at least in the anterior aorta, if not the circulatory system in general.

Possible route of delivery of the tetrapeptides to the heart. The tetrapeptides may be acting as neurotransmitters on the heart. They could be synthesised in the circumoesophageal ganglia and transported to the heart in neurones running in the visceral nerve and thence to the cardiac nerve. The cardiac nerve branches out, providing a diffuse innervation along the luminal surface of the heart. Here, the tetrapeptides would be released, in a high local concentration, from varicosities or terminals in close apposition to protected, postjunctional receptors. The tetrapeptides would thus be acting on the cardiac muscle at neuromuscular junctions.

Suggested route travelled by the heptapeptides from the brain to the heart. The heptapeptides are not present in the heart, but they have been detected at nanomolar levels in the blood. The aorta enters the suboesophageal ganglia, divides to supply the individual ganglia, then ramifies into the connective tissue sheath, eventually forming an almost continuous blood-filled space lying very close to the surface of the nervous

tissue (Pentreath & Cottrell, 1970). These data suggest that the heptapeptides could be neurohormones. If so, they would be secreted from the brain into the blood, and travel in the circulation to the heart. There they exert their effect via receptors that would be relatively exposed to the blood and particularly sensitive to the heptapeptides.

SCPs

Distribution of the SCPs within the central nervous system. The SCPs have been extracted from the brain and are also present at low levels in the heart. However, their distribution within the heart is unstudied, and their calcium-dependent release has yet to be demonstrated from any organ in Helix.

Immunohistochemistry of the molluscan central nervous system indicates that the SCPs are present mainly in the buccal ganglia; their levels in other central ganglia are small. The buccal ganglia of Helisoma exhibit positive staining with SCP antisera (Murphy, Lukowiak & Stell, 1985). Positive staining in the cerebral ganglia as well as the buccal ganglia has been demonstrated in Tritonia (Kempf, Masinovsky & Willows, 1987). Comparison of the immunoreactivity of the various ganglia in Aplysia brasiliana using RIA shows that SCP-immunoreactivity is highest in the buccal ganglia (75pmoles/pair), followed by the pedal (23pmoles), cerebral (16pmoles), pleural (9pmoles) and abdominal ganglia (6.8pmoles) (Lehman, Price & Greenberg, 1984). The buccal ganglia of Aplysia californica contain approximately ten times the amount of SCPs found in any of the other central ganglia (Lloyd, Mahon, Kupfermann, Cohen, Scheller & Weiss,

1985).

Presence of SCPs in the visceral and anal nerves. RIA analysis of HPLC fractions of the visceral and anal nerves combined indicates the presence of both SGYLAFPRMamide and MNYLAFPRMamide in these nerve trunks. In addition to innervating the gut, the visceral nerve also branches off into the cardiac nerve. Some of the SCP immunoreactivity may have been derived from the cardiac nerve; an HPLC/RIA analysis of the cardiac nerve alone might resolve this issue. Since however, this nerve branches off the visceral nerve quite close to the heart, two difficulties are presented. First, the dissection of this rather fragile piece of tissue would be difficult. Second, except for a very short length, the nerve is either within the main visceral trunk, or within the aorta (in which it appears to arborise very quickly to innervate the proximal anterior aorta and the heart).

Two hypotheses concerning the distribution of SCPs in the nervous system and their potency on the heart. The SCPs might travel from the brain to the heart by axonal transport. Alternatively or additionally, they might travel to the heart in the haemolymph.

The SCPs might travel from the brain to the heart by axonal transport. Transport could be via the cardiac nerve, but arborisation could be restricted until the axons reached the atrial-ventricular junction. There, they could then arborise and terminate non-synaptically. In the presence of an appropriate stimulus, the SCPs would be released, but their receptors might be on the luminal surface of the ventricle rather than within the atrial-ventricular junction.

Thus, the SCPs would arrive at the heart along a neural pathway, but would then act hormonally. This mode of delivery would be consistent with the low levels of the SCPs within the heart and also with the high sensitivity of the ventricle to them. As the receptors would be detecting SCPs carried in the blood, they would be exposed and sensitive to relatively low levels of peptide.

This proposal is supported by the description of a neurosecretory system in the atrial-ventricular junction of the Helix heart (Cottrell & Osborne, 1969); the vesicles in this neurohaemal site seemed unlikely to contain either 5-HT or dopamine. Moreover, the staining observed in this current study with the FMRFamide antiserum, S253, was not particularly dense in this region. To date, therefore, no transmitter has been positively linked with this atrial-ventricular neurosecretory system, but the SCPs are reasonable candidates.

Possible link between feeding and heart rate mediated by SCPs. An increase in heart rate associated with feeding has been observed in three molluscs: Aplysia californica (Dieringer, Koester & Weiss, 1978); Deroceras reticulatum (Duval, 1983); and Limax maximus (Grega & Prior, 1985). Lloyd, Kupfermann and Weiss (1985) have suggested that the link between feeding and increased heart rate may be partially mediated by SCPs spilling over into the blood from the digestive tract, but these peptides could not be detected in Aplysia haemolymph. However, neither was Lloyd able to detect SCPs in Helix heart using the same for bioassay (Lloyd, 1978a), though evidence has been presented here that two SCPs are extractable from Helix heart. The SCPs might enter the

circulation from the gut quite close to the heart. The peptides would then be at sufficient levels to excite the heart, but would be diluted by the haemolymph to undetectable levels downstream from the heart. This may also explain why Price (1987) was unable to detect SCP immunoreactivity in Helix haemolymph.

The immunohistochemistry, the RIA and the physiological data taken together suggest both hypotheses play a part in describing the physiological role of the SCPs in Helix. The SCPs may take a neural route directly to the heart, and also a circulatory route. Whatever the pathway followed, upon arriving at the heart they would then act as hormones.

Comparison of the Responses to the FMRFamide Analogues and the SCPs

Mechanisms of peptide action. What of the mechanisms by which these peptides act upon the heart? The responses induced by the FMRFamide analogues are clearly distinguishable from those induced by the SCPs, and the eight minute arrest of the ventricle by SGYLAFPRMamide did not appear to affect the subsequent FLRFamide response in figure 17D. Thus, these two groups of peptides probably act through different mechanisms.

The set of responses induced by the various FMRFamide analogues are relatively more uniform, but, they are not identical. For example, the response to pQDPFLRFamide (Fig. 17B) has more of a shoulder than that to FLRFamide (Fig. 17C), even at doses that are approximately equiactive. Similarly, the response to pQDPFLRFamide in figure 16B has a more rounded peak than the adjacent responses to FLRFamide. Finally, there are qualitative differences in all the pairs of responses shown in figure 18.

The slightly slower response of the ventricle to pQDPFLRFamide has also been noted by Price, Cottrell, Doble, Greenberg, Jorenby, Lehman and Riehm (1985).

In summary, these peptides are probably not all acting through the same mechanism and there could well be more than one component to a response as evidenced by the shoulders seen in the responses of figure 18.

Use of the patch-clamp technique. The cellular mechanisms of cardiac action of these peptides must be left to speculation if only the experiments described here are considered. Several issues have not been addressed: the ions involved in the responses to the different peptides; the site of peptide action whether on receptor-mediated ion channels or upon receptors linked to ion channels through second messengers, and the diversity of such agents.

The patch-clamp technique (Hamill, Marty, Neher, Sakmann & Sigworth, 1981) may be able to answer some of these questions. First the ventricular and atrial cells would have to be dissociated separately. Whole-cell recording from these cells would quickly show if the peptides affected the membrane current or potential. If so, application of the peptides to isolated patches would allow the channels affected by each peptide to be characterised, and bath application of the peptides to cells in the cell-attached patch configuration would reveal whether any second messengers were involved. If so, these second messengers could be identified by the use of isolated patches. The ionic mechanisms by which each peptide effected its response could be determined by almost any combination of the patch configurations

once the channels affected by each peptide had been identified.

Voltage clamp techniques have been used successfully on dissociated leech heart muscle cells; they are depolarised by as little as 10^{-12}M FMRFamide in the bathing saline (Thompson & Calabrese, 1988). Preliminary results seem to implicate the involvement of calcium in the response.

Physiological Significance of the Different Peptides

Do the effects of these peptides on the isolated ventricle and their distribution within the central nervous system tell us anything about their physiological significance?

The heptapeptides. There are five heptapeptides present in Helix and all are equipotent on the isolated ventricle from aestivating snails. This finding is not consistent with those from a study of the potencies of a series of peptides on the radula protractor of Busycon contrarium and the heart of Mercenaria mercenaria (Greenberg, Payza, Nachman, Holman & Price, 1988). FMRFamide was assigned a value of 1.0, and all the other peptides were standardised against FMRFamide. When the N-terminal phenylalanine was replaced by a tyrosine (YMRFamide; i.e., an hydroxyl group was added), the potency fell from 1.0 to 0.3 (radula protractor muscle) and from 1.0 to 0.14 (Mercenaria heart). However, a similar change in the heptapeptide sequence (i.e., from NDPFLRFamide to NDPYLRFamide) did not affect the potency of the peptides on the Helix ventricle.

These contrasting observations -- the differing potencies of the tetrapeptides on the two muscles vs. the equipotency of the heptapeptides on the Helix heart -- suggest that the radula protractor and the clam heart have receptors different to those

of the Helix heart. Alternatively, this change is a terminal change for the tetrapeptides (occurring at the N-terminal end of the peptide) where it might be rather exposed and so important to the receptor while the change is in the middle of the hepta-peptide where it might be masked and not be so important to the receptor. That is, the heptapeptide receptor may only require an aromatic residue in this position and it may not matter whether this residue is phenylalanine, tyrosine or tryptophan. The appropriate tyrosine heptapeptides have not been tested on the radula protractor muscle or the clam heart and only the endogenous tyrosine analogues have been tested on the isolated Helix ventricle. Perhaps other Helix muscles or neurones can distinguish between these five heptapeptides.

Comparison of the relative potencies of FMRFamide and FLRFamide on ventricles from aestivating and active animals.
All the peptide pairs were initially tested on ventricles removed from animals that had been aestivating for several months. However, one peptide pair was also tested on ventricles removed from animals that had been active for at least a week immediately before their removal. The responses of ventricles from active and aestivating Helix to FLRFamide and FMRFamide are qualitatively similar, but quantitatively different. The order of potency is the same, but the potency difference is 11.694 +/- 2.63 (mean +/- s.e.m.) for aestivating animals and 4.89 +/- 0.623 for ventricles from active animals. This difference is significant at $p<0.001$ using the Student's T-test. Thus, the relative sensitivity of the isolated Helix ventricle to FMRFamide and FLRFamide is partly dependent upon the state of the animal from which it is removed.

The mechanism behind this observation is not known at present.

Relative levels of FMRFamide analogues and state of activity. Lehman and Greenberg (1987) have proposed that the relative levels of FMRFamide and pQDPFLRFamide in Helix blood, as well as the antagonistic actions of the peptides, determine the state of activity of the animal. In short, rising levels of pQDPFLRFamide arouse the animal while rising levels of FMRFamide cause it to withdraw into its shell. Further investigations of this hypothesis should include a comparison of the relative potencies of the peptides on ventricles from active animals, and on their vasculatures, and more precise data on the levels of the individual peptides in the haemolymph of animals in various states of activity.

Haemolymph samples from different regions of the circulation might provide further information regarding the distribution, if any, of the SCPs in the haemolymph. An alternative method for determining if circulating levels of SCPs are affected by the presentation, ingestion or digestion of food with concomitant stimulatory actions on the heart, would be to inject SCP antibodies into the circulation at the time of presentation of food. Presumably circulating SCPs would be bound and changes in the action of the heart in the presence and absence of the circulating antibodies could be measured. This would not be a particularly easy experiment, and some of the other protocols suggested or outlined above would also require much patience, imagination and determination. However if they helped to elucidate the physiological roles and mechanisms of action of these peptides in Helix, the effort would have been substantially repaid.

CHAPTER TWO

ACTIONS OF FMRFamide ANALOGUES ON THE SYSTEMIC VENTRICLE OF *Eledone cirrosa*, AND THEIR POSSIBLE PRESENCE IN THE CENTRAL NERVOUS SYSTEM

Introduction

Background. Molluscan cardioregulation has attracted the interest of experimental biologists for more than a century, and the cephalopod heart and circulation have been among the topics investigated. Early work focused on the comparative anatomy and innervation of the hearts (Ransom, 1884; Fry, 1909). Much later, Alexandrowicz noted the unusually dense innervation of the anterior vena cava, and further anatomical investigation prompted him to suggest a neurosecretory role for it in *Eledone cirrosa*, *Sepia officinalis* and *Octopus vulgaris* (1964, 1965).

Bioactivity of cephalopod extracts. Berry and Cottrell (1970) prepared extracts of the anterior vena cava from *Eledone cirrosa* and applied them to the isolated systemic heart of the same species. The response was an increased amplitude and frequency of beat. They found that the activity was heat stable, and not attributable to 5-HT, adrenaline or nor-adrenaline.

Blanchi, Noviello and Libonati (1973) extended these experiments, testing vena cava extracts from several cephalopods on the isolated hearts. Cardioactive extracts could be isolated from every species investigated. The extracts were stable to heat and pronase, but not to trypsin or papain. Moreover, an extract from any of the three octopods investigated (*Octopus vulgaris*, *O. macropus* and *Eledone moschata*) was equally effective on the hearts of all three species. The medium bathing the isolated venae cavae became cardioactive when the organ was electrically stimulated, and the response of the hearts to these

extracts was similar to the response to the "chemically isolated" extracts. Extracts of the vena cava also stimulated the hearts and increased the blood pressure of intact Octopus vulgaris (Wells & Mangold, 1980).

Immunohistochemistry. Immunohistochemistry was called into play next. Martin, Froesch, Weber and Voigt (1979) noted met-enkephalin-like immunoreactivity in the neurosecretory region of the Octopus vena cava; they reasoned that the staining was quite specific. Positive staining has since been observed in the vena cava with antisera raised against alpha-melanotropin, arginine-vasopressin (Martin, Froesch & Voigt, 1980), FMRFamide (Martin, Froesch, Kiehling & Voigt, 1981), proctolin, oxytocin, neurophysin I and II, rat atriopeptin II (ANF) and leu-enkephalin (Martin & Voigt, 1987).

Objectives. FMRFamide had not been sequenced when Berry and Cottrell were investigating the effects of vena cava extracts on the isolated systemic heart of Eledone, but the extract appeared to be peptidic and Octopus nervous tissues were positively stained with FMRFamide antiserum. Therefore, an investigation of the effects of FMRFamide and its analogues on the isolated systemic heart of locally available Eledone cirrosa was initiated. Additionally, tissue extracts were collected for initial analysis by HPLC and RIA; the aim was to identify the active substances of the vena cava and other tissue extracts.

Methods

Dissection of Systemic Heart and Tissues for Biochemical Analysis

Dissection of heart and vena cava. Eledone cirrosa were caught in the North Sea off the Fife coast. They were transported to the Gatty Marine Laboratory in covered, water-filled containers, transferred to covered aerated tanks, and allowed a few days to recover from their ordeal. As each animal was required, it was recaptured, transferred to the experimental laboratory, left to rest for thirty to sixty minutes, and anaesthetised with magnesium chloride solution (Messenger, Nixon & Ryan, 1985). The mantle septum was quickly severed so that the mantle could be reflected to expose the internal organs.

The vena cava was transected immediately exterior to the cartilage enveloping the brain, and the brain in its case was severed from the rest of the body and set aside (see next section). The vena cava was dissected free for approximately three-quarters of its length to the branchial hearts. It was then weighed and placed in a stoppered container with four times its mass of acetone.

The systemic heart was then carefully dissected free; the thin pericardial membrane was removed from around the ventricle. The left efferent branchial vessel was cannulated, the tip of the cannula being within the ventricle itself. The right branchial vessel was ligated, and the smaller gonadal artery and abdominal aorta were either severed at a distance from the heart so that they folded and sealed shut, or were ligated. The anterior aorta was freed for a length of approximately two centimeters from the

heart. An incision was made in the wall severing it halfway and a length of strong cotton thread tied in this nick so that the vessel was not blocked to the flow of liquid. The aorta was severed distal to this tie. (Nomenclature is that of Isgrove (1909).)

The cannula with its tip in the ventricle was connected to the perfusion system with a silicone tube so that the heart was freely suspended (Fig. 22). The flow of decanted sea water was initiated and set at a rate just fast enough to permit a small overflow to bath the external surface of the heart. The thread from the anterior aorta was fastened to a drinking straw which magnified the beat. At the far end of this straw was a glass pen which could be positioned against the side of a smoked drum to record the rate and amplitude of the beats.

Dissection of the brain and optic lobe for analysis. While the ventricle was left to beat and equilibrate for thirty minutes, the main brain and optic lobes were dissected from their cartilaginous case. They were weighed separately and placed in separate stoppered containers with four times their mass of acetone. These tissues were then stored at -20^{o}C until required for HPLC.

Comparison of the Effects of the Peptides on the Isolated Ventricle Apparatus. Sea water flowed from a reservoir through silicone tubing almost to the heart (Fig. 22). Just before arriving at the heart, the tubing was interrupted by a glass Y-piece. The tubing was connected to one of the top arms and the bottom arm. A short length of tubing connected the bottom arm to the cannula. The purpose of the second top arm was twofold; it maintained a constant head of pressure because it was open to the

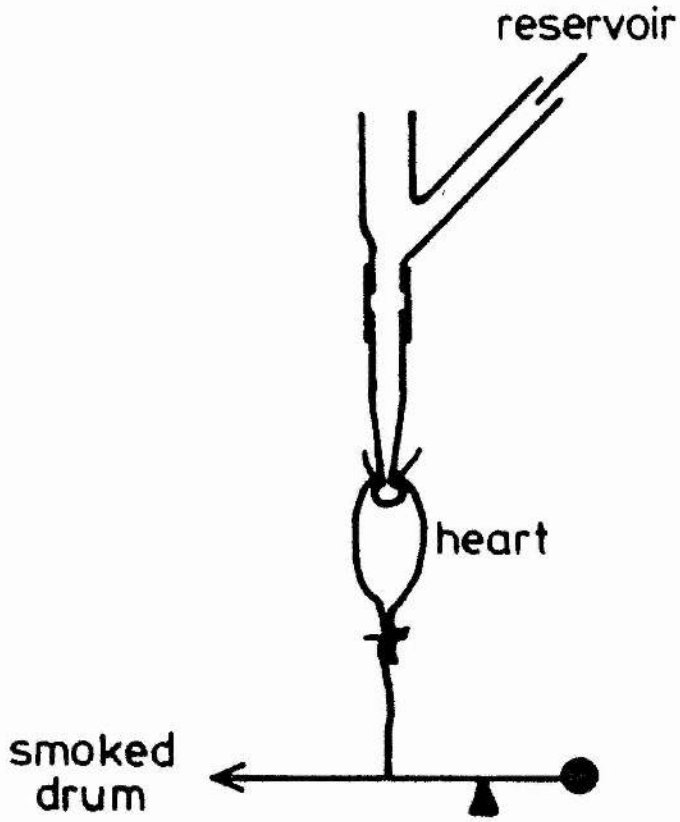


Figure 22. A diagram of the open tubing arrangement for the comparison of the potencies of some FMRFamide analogues on the isolated Eledone ventricle. The peptides were injected as a bolus through the wide vertical tube which was open to the air and thus at atmospheric pressure.

atmosphere, and it was through this arm that each bolus of peptide was injected.

Application of the peptides. Stock solutions of peptide at a concentration of no less than 10^{-5}M in decanted sea water were made and stored at -20°C. For testing, serial tenfold dilutions were made to the required concentration. At ten minute intervals, a bolus of 0.1, 0.2 or 0.4ml peptide solution was injected into the Y-piece. Nineteen gauge, 1.5 inch needles were used so that the injection could be made rapidly and almost directly into the lumen of the ventricle. Care was taken to disturb the preparation as little as possible.

Although the systemic heart was removed from the animal, only the ventricle could respond to the peptides. The cannula was inserted straight through the left atrium into the ventricle and the right atrium was ligated with the right efferent branchial vessel.

Optimum size of animal. The carcass was weighed and comparison of the performance and life span of the isolated heart with the mass of the animal from which it was removed revealed that the most reliable and useful hearts were those from animals with a mass of 200-400g.

Detection of immunoreactive peaks with FMRFamide antisera

The three vials with their tissues were transported to the Whitney Laboratory in a vacuum flask filled with dry ice. There, the acetone was decanted off and the tissue washed with several volumes of acetone. The acetone was pooled with the dissolved extract. The acetone was then evaporated off with a rotary evaporator connected to a water aspirator in a water bath heated to 65°C. The remaining liquid was diluted with HPLC buffer A

and centrifuged to sediment any particles. The extract was separated by HPLC, and the fractions tested for immunoreactivity with the S253, Q2 and SCP_B antisera as for the Helix tissue.

Results

Responses of the Isolated Ventricles to the Peptides

Shape of response. All of the FMRFamide analogues tested stimulated the isolated ventricle (Fig. 23); an initial sharp increase in the beat amplitude was often followed by a secondary shoulder that was more slow to develop and decline. The responses shown have been chosen for their equipotency, and both members of a pair are from the same ventricle, but not all the responses were qualitatively similar. The response to a bolus of FLRFamide had a more obvious shoulder than that to FMRFamide (Fig. 23A). The shoulders of the responses to the extended analogues were more slow to develop than those of the responses to FMRFamide (Fig. 23B and C); the shoulder of the NDPFLRFamide was the slowest to develop (Fig. 23C). The rapid fading of the responses in figure 23B was probably more a function of that particular ventricle (it had a very shallow beat) than the peptides. Thus, while the responses of that ventricle to FMRFamide and YGGFMRFamide can be compared, comparison of these two particular responses with those from another ventricle would be inappropriate.

Relative potencies. The relative potency differences of the peptides on the isolated systemic ventricles are summarised in figure 24. In short, extending the peptides at the N-terminal, from four residues to seven, decreased their potency on the ventricle. The greatest potency difference (a factor of 240) is that between FMRFamide and the extended leucine analogue, NDPFLRFamide; FMRFamide is only four times more potent than FLRFamide. As with the Helix ventricle, not all the peptides

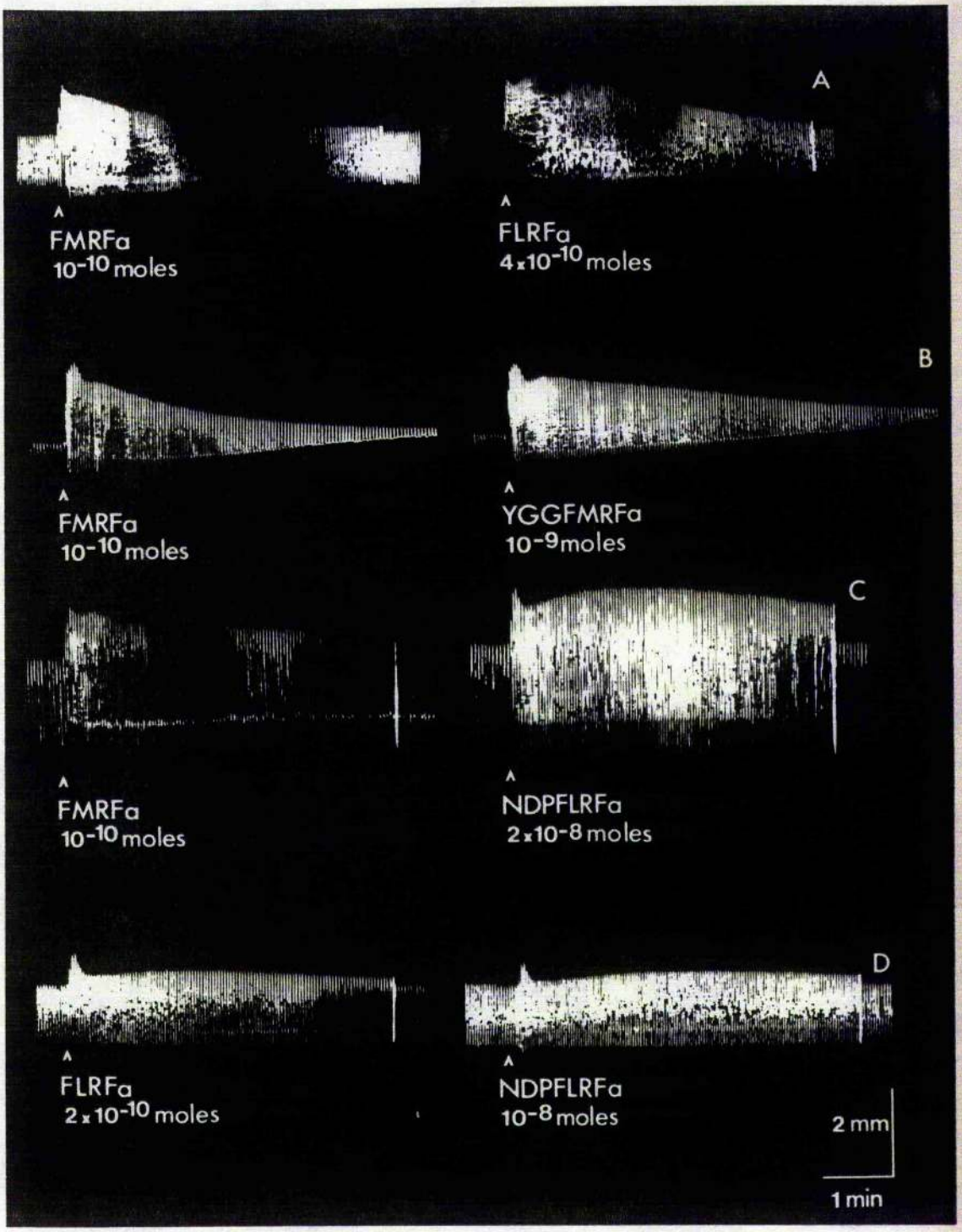


Figure 23. Pairs of approximately equipotent doses of peptides applied to the isolated ventricle of Eledone cirrosa. The peptides were applied at the arrows as a bolus containing the number of moles of the peptide indicated below each dose. Each of the four records is from a different ventricle.

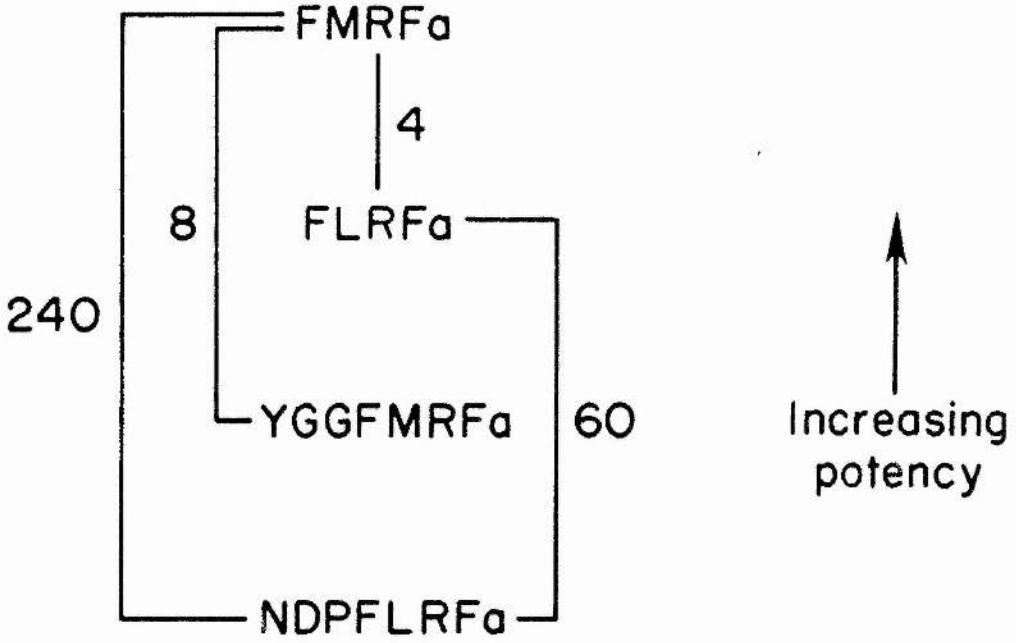


Figure 24. The relative potencies of the peptides on the isolated ventricle of Eledone. The order of potency decreases from top to bottom.

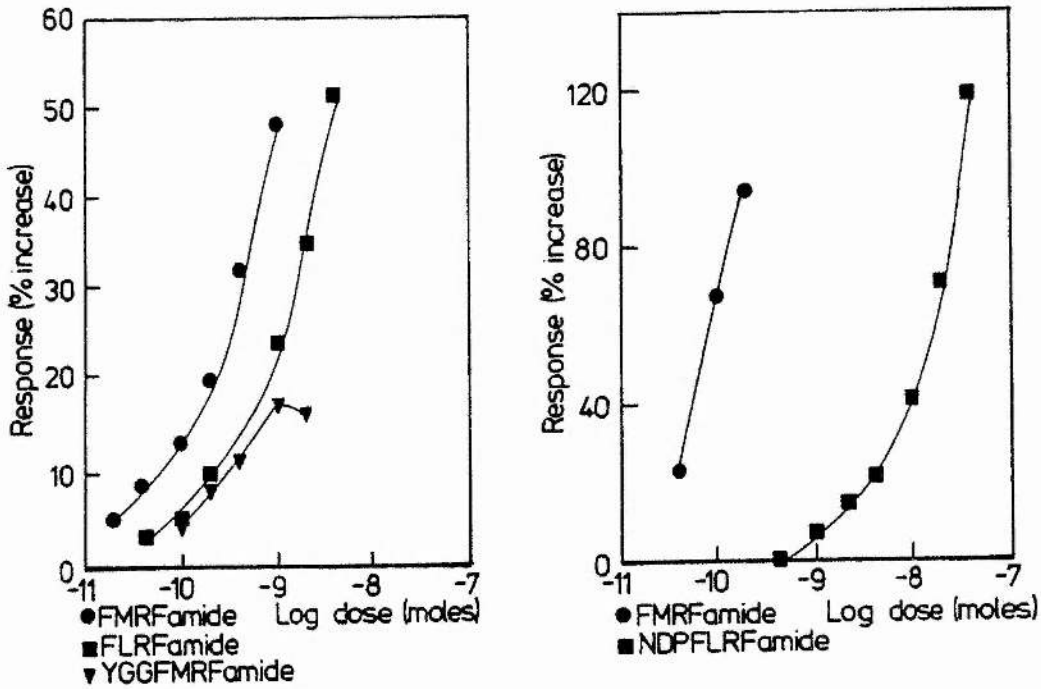


Figure 25. Two sets of dose-response curves for the peptides on the isolated ventricle of Eledone. All curves for a set are from the same ventricle, but each set is from a different ventricle.

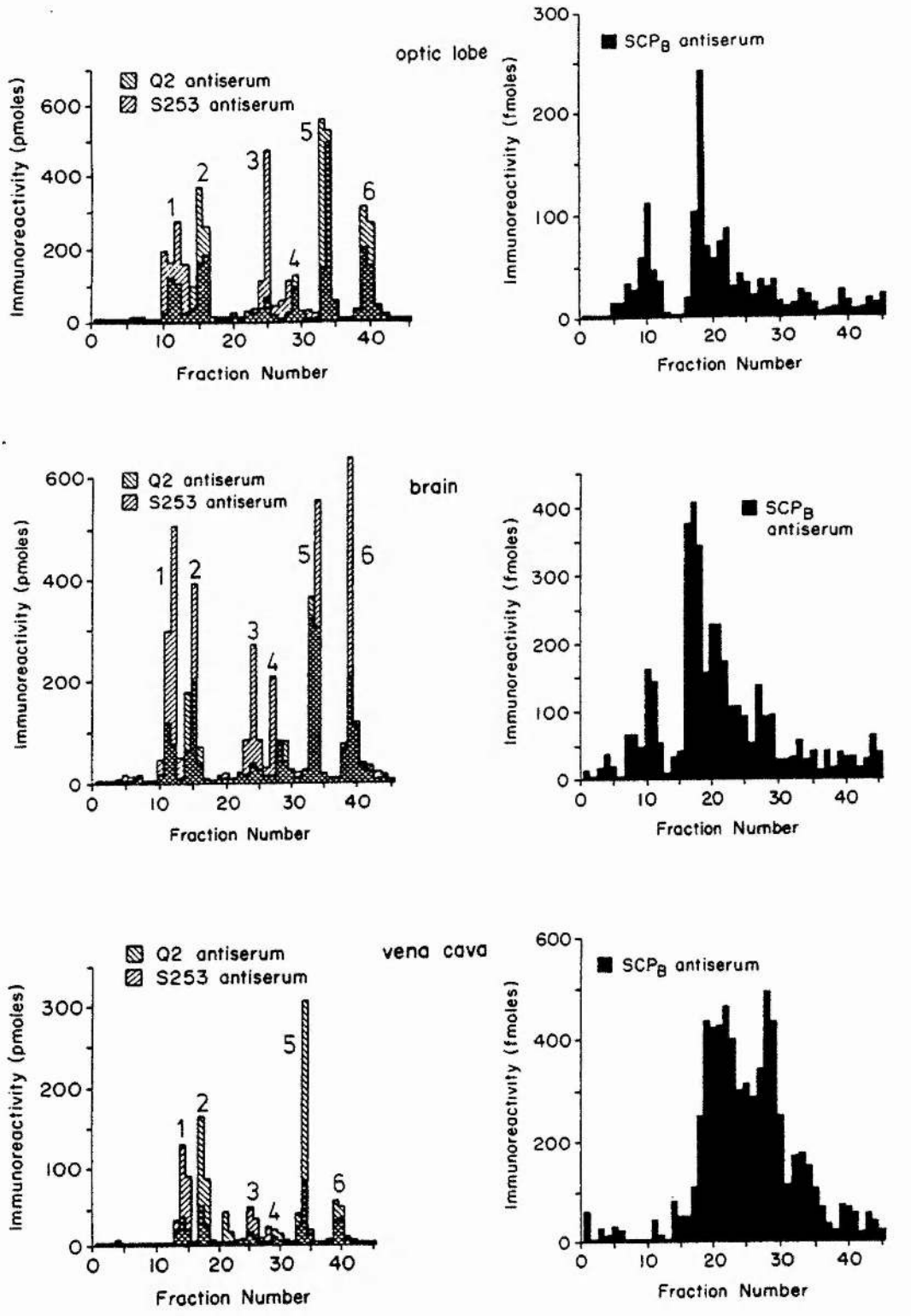


Figure 26. RIA analysis of HPLC fractions of acetone extracts of the optic lobe (top), brain (middle) and vena cava (bottom) from Eledone cirrosa. The peaks identified with S253 are superimposed upon those identified with Q2 (left). The same fractions are analysed with SCP_B antiserum (right).

evoked the same maximum response from a given ventricle (Fig. 25). Thus, the relative potencies of the peptides on the Eledone ventricle were determined by comparing equipotent doses.

All four peptides were tested on a minimum of three ventricles. FMRFamide and FLRFamide, being the most potent, were compared on seven ventricles. However, only three ventricles were sensitive enough to respond to more than just the highest dose of NDPFLRFamide.

RIA Analysis of the Extracts

All three tissues displayed six peaks immunoreactive to both S253 and Q2; the peaks had similar elution times on the same HPLC gradient (Fig. 26). Because the six peaks in all three tissues were identified by both S253 and Q2, FMRFamide, or one or more of its analogues, is probably present, though this is not certain. Comparison of the Eledone peaks with those of Helix indicates that the first two Eledone peaks could be FMRFamide and FLRFamide, respectively. The two Octopus pentapeptides, AFLRFamide and TFLRFamide, could also be present in Eledone, though their elution times with the HPLC gradient used here are not known. The fractions concerned have been saved, and potential analogues synthesised; after further purification the relevant peptides will be identified by fast atom bombardment spectroscopy and sequence analysis.

The peaks identified with SCP_B antiserum were less distinct and their level of immunoreactivity was very low (Fig. 26) so they have been discounted for the present as their validity is in doubt.

Discussion

Responses of Cephalopod Hearts to FMRFamide Analogues

Eledone ventricles. All the FMRFamide analogues tested elicited responses from the isolated systemic ventricle of Eledone. However, they were not equipotent, nor were their effects identical. The order of potency of the FMRFamide analogues on the Eledone ventricle is the reverse of that on the Helix ventricle, but the significance of this relationship remains obscure.

Comparison of Octopus and Eledone. FMRFamide, FLRFamide, YGGFMRFamide and YGGFLRFamide all had positive effects on the frequency and amplitude of the beat of the isolated systemic heart of Octopus vulgaris (Voigt, Kiehling, Froesch, Schiebe & Martin, 1981). The frequency effects of the analogues tested on the Eledone ventricle were, however, slight and secondary to the amplitude effect.

Mechanism of the responses. Two qualitative differences in the responses were observed: the FLRFamide-evoked response had a more prominent shoulder than that of FMRFamide; and the shoulders on the responses elicited by the extended analogues were slower to develop than those elicited by FMRFamide. These differences suggest a diversity of mechanisms of action; e.g., different ionic channels, second messengers, or other intracellular mechanisms could be involved. However, the results, though intriguing, are insufficiently developed to support speculation. As with the Helix ventricle, further experiments are required.

FMRFamide analogues endogenous to cephalopods. All the FMRFamide analogues tested on the ventricle or other tissues of Helix are, indeed, endogenous to it. In contrast, information about Eledone, whether biochemical or pharmacological, is incomplete.

Octopus vulgaris has been the most popular cephalopod for the type of experiments discussed here. This may be due, in part, to its larger size and its greater availability to those interested in cephalopods. Peak C (the impure "precursor" of FMRFamide) was isolated from the brain, optic lobes and systemic heart of Octopus bimaculatus (Agarwal, Ligon & Greenberg, 1972). More recently, FMRFamide, FLRFamide, AFLRFamide, TFLRFamide and YGGFMRFamide have been identified in extracts of the nervous tissue of Octopus vulgaris (Martin & Voigt, 1987; Voigt & Martin, 1986), though the authors have some reservations concerning YGGFMRFamide (Martin & Voigt, 1987).

Care should be taken when drawing analogies between Octopus and Eledone. The identification of a peptide in one species does not ensure its presence in another. For example, GDPFLRFamide is to be found in Siphonaria (Price, Cobb, Doble, Kline & Greenberg, 1987), but not in Helix.

Immunoreactive Fractions from Eledone Tissue

This investigation of the actions of the FMRFamide analogues on the Eledone systemic ventricle began before data was published concerning the possible presence of these analogues in Octopus. Two goals were in mind at that time: the effect of these peptides on the ventricle; and (to put the cardioregulatory data in perspective) the distribution of these peptides in the vena cava

and central nervous system of Eledone. Thus the brains (minus the optic lobes), the optic lobes and venae cavae were collected for acetone extraction and subsequent RIA of the HPLC fractions.

Six immunoreactive peaks seem common to all three tissues. The fractions composing the six peaks immunoreactive to both S253 and Q2 have been saved for purification and identification. The SCP immunoreactivity seemed too low to pursue. Little more can be said about the source of this immunoreactivity with the information currently available.

Possible Roles for FMRFamide Analogues in Cephalopods

Main brain and vena cava. Let us for the moment assume that FMRFamide or its analogues are present in these tissues. What could be the possible significance of their presence in all three tissues? Their presence in the brain could be explained in part by their presence in the vena cava. The vena cava is a neurohaemal release site; the neuronal somata reside in the brain, the axons project to the vena cava. The peptide precursors are probably synthesised in the brain; the peptides are then processed as they are transported along the axons.

What of their presence in the vena cava? The cardioactivity of the peptides has been demonstrated, and the vena cava is a reasonable release site for a substance with such a role. The brain could receive the sensory information indicating a need for increased cardiac output and so stimulate the release from the vena cava of one or more of these peptides which would be carried in the blood to the systemic heart producing the required stimulation.

An additional role for FMRFamide has been suggested by experiments performed with Sepia officinalis. Le Gall, Féral, Van Minnen and Marchand (1988) have noted FMRFamide-immunoreactive fibres originating in the basal-dorsal lobe and the olfactory lobe of the central nervous system. These fibres form an extensive network in the inferior part of the dorsal-lateral lobe and traverse through the optic tract to innervate the optic gland. These lobes are involved in the integration of chemical and visual stimuli and the control of reproduction. The identity of the antigen is not yet known; nor has FMRFamide itself been tested in this system, but the possibility remains that this peptide has a role in the control of reproduction in cephalopods.

Optic lobes. What, however, could be the significance and purpose of these peptides in the optic lobe? The pharyngeal-ophthalmic vein supplies the eye and related tissue. Froesch and Mangold (1976) tested extracts of the pharyngeal-ophthalmic veins from Eledone on systemic hearts of the same. The extracts were cardioactive, but their effects, compared with those of FMRFamide and its analogues, were distinctly dissimilar. The extracts were, of course, not pure but were mixtures of compounds, any number of which could be cardioactive. Thus, although FMRFamide or one of its analogues might well have been present, its effect would have been grossly modified by those of other cardioactive constituents of the extract.

Boycott and Young (quoted in Froesch & Mangold, 1976) suggested that the subpendiculate nerve might be involved in regulating blood pressure in the eye cavity. Although Froesch and Mangold supported this theory in principle, they suggested it

might be too restricted, and that this regulatory activity might be more widespread within the animal.

FMRFamide immunoreactivity associated with eyes in other animals. Positive staining with FMRFamide antisera has been observed in the eyestalks of the prawn (Jacobs & Van Herp, 1984) and crayfish (Van Deijnen, Vek & Van Herp, 1985), and retinal cells of the perch (Zucker & Dowling, 1987), goldfish (Stell, Walker, Chohan & Ball, 1984; Muske, Dockray, Chohan & Stell, 1987) and frog (Wirsig-Wiechmann & Basinger, 1988). Thus FMRFamide could be involved in the control of blood pressure in the optic cavity, and the body in general (important for a cephalopod which may alter its depth in the water), but it could also act as a general ophthalmic transmitter, as suggested for crustaceans.

Other Cardioactive Peptides That May Be Associated with Molluscs

The earlier assumption that one or more FMRFamide analogues were present in Eledone need not preclude the presence of other cardioactive peptides as well. Four mammalian atrial peptides: cardiodilatin 1-16, atriopeptin I and II, and atrial natriuretic factor 8-33, all affect the systemic heart of Octopus vulgaris (Agnisola, Cariello, De Santis, Miralto & Tota, 1989). As yet, there is no evidence for their presence in any cephalopod, though positive staining with cardiodilatin antiserum has been observed in the mollusc Helix pomatia, and atrial and suboesophageal extracts relaxed the smooth muscle of rabbit aortic strips in a dose-dependent manner (Reinecke, Nehls & Forssmann, 1986).

Other Molluscan Peptides

Catch-relaxing peptide (Ala-Met-Pro-Met-Leu-Arg-Leu-NH_2), sequenced from pedal ganglia extracts of Mytilus edulis, relaxes catch tension of the anterior byssus muscle of Mytilus (hence its name) (Hirata, Kubota, Takabatake, Kawahara, Shimamoto & Muneoka, 1987). This peptide also inhibits cardiac contractions in Mytilus and two other bivalves (Meretrix lusoria and Tapes japonica).

Histidine-rich basic peptide, 43 amino acid residues long, N-terminally blocked, and obtained by molecular genetics from Aplysia californica neurones R3-14, induces a dose-dependent increase in beat amplitude of the isolated heart and isolated ventricle, while the isolated atrium responds with increased beat amplitude and frequency (Campanelli & Scheller, 1987).

Three other peptides have been sequenced from Aplysia californica. Myomodulin (Pro-Met-Ser-Met-Leu-Arg-Leu-NH_2) and buccallin (Gly-Met-Asp-Ser-Leu-Ala-Phe-Ser-Gly-Gly-Leu-NH_2) have been isolated from two neurones that modulate the accesory radula closure muscle (Cropper, Tenenbaum, Kolks, Kupfermann & Weiss, 1987; Cropper, Miller, Tenenbaum, Kolks, Kupfermann & Weiss, 1988). Pedal peptide (Pro-Leu-Asp-Ser-Val-Tyr-Gly-Thr-His-Gly-Met-Ser-Gly-Phe-Ala) isolated from the pedal ganglia, has a free carboxyl terminal (Lloyd & Connolly, 1989). Pedal peptide induces net inward currents associated with a decrease in membrane conductance in neurones L2, L3 and L5. As the main interest of the two laboratories responsible for these three peptides is not cardiac physiology, these peptides have not yet been tested on hearts.

Thus all the FMRFamide analogues tested on the isolated systemic ventricle of Eledone cirrosa are cardioactive. One or more of them may be endogenous to this animal, and so may other cardioactive compounds. The identity of the endogenous cardioactive substances remains to be demonstrated, as do their precise mechanisms of action and their physiological roles.

CHAPTER THREE

COMPARISON OF THE EFFECTS OF FMRFamide ANALOGUES ON THE CALCIUM CURRENT OF THE C1 NEURONE OF *Helix aspersa*

Introduction

Actions of FMRFamide Analogues on Helix Neurones

Comparison of effects of FMRFamide analogues on the ionic currents of Helix neurones. Cottrell, Davies and Green, (1984) and Cottrell and Davies, (1987) compared the ionic effects of FMRFamide and some endogenous and synthetic analogues on identified and unidentified neurones of the central ganglia of *Helix aspersa*. Two main points arose from their investigations. First, four different responses could be evoked by the various analogues, but the two groups of analogues (tetrapeptides and heptapeptides) were not equipotent for any of these responses. Second, a brief survey with the analogues employed revealed certain minimum structural requirements for activity. In particular, the C-terminal -Phe-amide was required and N-terminal extension altered the properties of the peptides, increasing the potency for some effects and decreasing it for others.

The response evoked by a particular peptide depended upon the particular neurone to which it had been applied and the holding potential of that neurone. Some neurones could exhibit more than one response (depending upon the peptide and the holding potential). In summary, one sodium-dependent response and three potassium-dependent responses were seen. One potassium-dependent response was due to a voltage- and calcium-dependent decrease in the conductance. The remaining two potassium-dependent responses and the sodium-dependent response were caused by increases in ionic conductance.

Comparison of effects of FMRFamide and pQDPFLRFamide on Helix neurones. Boyd and Walker (1985) examined the effects of FMRFamide on a range of neurones in the suboesophageal ganglia of Helix aspersa. Cells that were depolarised by ACh were hyperpolarised by FMRFamide. Experiments manipulating the external potassium concentration and the addition of 4-AP to the bath suggested this was due to an increase in potassium conductance.

Conversely, cells that were hyperpolarised by ACh were depolarised by FMRFamide. Removal of sodium from the bath saline abolished the FMRFamide response and hyperpolarising current pulses were increased in the presence of FMRFamide. Thus, this response was mediated by an increased sodium current in these cells. D-tubocurarine, tetrodotoxin, morphine, naloxone and met-enkephalin were all without effect.

Cottrell and Davies (1987) found that FMRFamide and FLRFamide were equipotent in their effects on the neurones they tested (though FIRFamide was sometimes slightly less potent than the other two tetrapeptides). Boyd and Walker (1985) concurred with them where hyperpolarising (increased potassium conductance) responses were concerned, but differed in their findings with sodium responses. "FLRFamide was consistently less potent than FMRFamide" in its effect on the F77 cell, though cross-desensitization was evident implying that both peptides were acting through the same receptor. Cottrell and Davies (1987) also observed that the response mediated by increased sodium conductance was desensitized by the three tetrapeptides.

Boyd and Walker (1987) went on to compare the effects of FMRFamide and pQDPFLRFamide on six identified neurones in the

suboesophageal ganglia of Helix aspersa. pQDPFLRFamide was unable to depolarise any cell tested, even one that was depolarised by FMRFamide. Generally, pQDPFLRFamide was less potent in its effect upon a cell than FMRFamide. Occasionally they were equipotent, as they were on cell E13. Here both peptides hyperpolarised the cell, suggesting that the E13 neurone described by Cottrell and Davies (in which FMRFamide appeared to induce a depolarising sodium current while pQDPFLRFamide was apparently without effect) was not the E13 described by Boyd and Walker. FMRFamide and pQDPFLRFamide could induce opposing effects on the same neurone as they did for F77 where FMRFamide depolarised the cell while pQDPFLRFamide hyperpolarised it.

Effect of FMRFamide on other currents in Helix neurones. A third laboratory developed an interest in FMRFamide and its effects on identified neurones in the suboesophageal ganglia of Helix aspersa. Colombaioni, Paupardin-Tritsch, Vidal and Gerschenfeld (1985) noted the effect of FMRFamide on yet a fourth potassium current. FMRFamide was observed to decrease a potassium conductance that was not dependent upon intracellular free calcium levels (as it was not affected by intracellular injections of the calcium chelator EGTA). Instead, it was mediated via cAMP (as judged by the effects of cAMP, forskolin and IBMX upon the E11 neurone). Intracellular injection of cAMP could mimic the actions of FMRFamide on the E11 neurone. Forskolin (a diterpene activator of adenylate cyclase) and the phosphodiesterase inhibitor IBMX both evoked inward currents which could not be increased further by FMRFamide. FMRFamide also stimulated adenylate cyclase activity in this neurone.

Combining this evidence the authors proposed that FMRFamide was decreasing a cAMP-mediated potassium current in the E11 neurone, and that this current was similar to the S-current of Aplysia sensory neurones described by Klein, Camardo and Kandel (1982).

FMRFamide also decreased a calcium current in the D3, E2 and E13 neurones, probably through a second messenger mechanism, but not one involving an increase in intracellular cAMP, cGMP or calcium levels.

Actions of FMRFamide and YGGFMRFamide on Aplysia neurones. FMRFamide and YGGFMRFamide are both equipotent in suppressing a voltage-dependent calcium current in particular neurones of Aplysia californica (Brezina, Eckert & Erxleben, 1987b). They were only able to suppress 30-50% of the total calcium current. Though evidence suggested that this suppression was mediated via a second messenger, such a messenger has not yet been identified.

While FMRFamide appears to mimic the action of 5-HT on the S-current in Helix neurones, it opposes the action of 5-HT on neurones in Aplysia californica (Belardetti, Kandel & Siegelbaum, 1987; Brezina, Eckert & Erxleben, 1987a). Patch-clamp experiments have shown that 5-HT reduces the background current in Aplysia by a cAMP-dependent closure of S-channels in an "all-or-none" manner. FMRFamide (and YGGFMRFamide at equimolar concentrations), however, can reverse the closure of these channels by 5-HT or cAMP. They increase the probability of each S channel opening using a second messenger that is not cAMP.

A second difference in the action of FMRFamide on potassium currents in Aplysia as compared to Helix was also noted by Brezina, Eckert and Erxleben (1987a). While FMRFamide and YGGFMRFamide

seem to directly suppress the calcium-dependent potassium current in Helix (Cottrell, Davies & Green, 1984), in Aplysia this suppression is indirect (Brezina, Eckert & Erxleben, 1987b). FMRFamide and YGGFMRFamide have no effect on the calcium-dependent potassium current which has been activated by calcium injection under voltage clamp; the calcium current is not activated under these conditions. However, the peptides do reduce the calcium-dependent potassium current which has been evoked by depolarisation and hence calcium influx. Thus FMRFamide and YGGFMRFamide are suppressing the calcium current which, in turn, suppresses the calcium-dependent potassium current; the peptides are not acting directly on the potassium current itself. So comparisons between Helix and Aplysia need to be made with caution.

The actions of FMRFamide on three identified neurones in the left upper quadrant of the abdominal ganglion of Aplysia have been investigated by a fifth laboratory. FMRFamide elicits a biphasic response from the burster neurones L4 and L6. An initial increase in the sodium conductance is followed by a subsequent increase in a conductance whose reversal potential resembles that of potassium currents. Though this second current is neither fast nor particularly transient, it is potassium-dependent, unaffected by 100μM TEA, but blocked by 40-90% by 1mM 4-AP in the bath saline; the same concentration of 4-AP also blocked 50-100% of the potassium A current in L4 and L6 (Ruben, Johnson & Thompson, 1986).

FMRFamide activates an inward rectifier current in neurone L2 which is dependent upon the intracellular chloride concentration (Thompson & Ruben, 1988).

Table 4 summarises the currents in Helix aspersa and Aplysia californica that are affected by FMRFamide or close analogues. Comparisons have been made between the effects of FMRFamide and some close analogues on the sodium and potassium currents of Helix. We turn now to a comparison of the action of FMRFamide, FLRFamide, and a heptapeptide analogue of each, on the calcium current of the C1 neurone of Helix aspersa. Some of these results have been previously published in abstract (Cottrell & Lesser, 1987).

Helix	1 gK slow	1 gK fast	1 gNa	1 gK_V	2 gCa	2 gK_S
FMRFamide	+ +	N E	+ +	-	-	- -
FLRFamide	+ +	N E	+ +	-	x	x
FIRFamide	+ +	N E	+ +	-	x	x
pQDPFLRFamide	+	+ +	N E	- -	x	x
YGGFMRFamide	+	+ +	N E	- -	x	x

Aplysia	3 $gK_{A?}$	3 gNa	4 gCa	4&5 gK_S	5 gK_{Ca}	6 gCl
FMRFamide	+ +	+ +	-	+ +	-	+ +
YGGFMRFamide	x	x	-	+ +	-	x

Table 4. A summary of the ionic currents affected by FMRFamide and its analogues in Helix aspersa (top) and Aplysia californica (bottom). The number of + signs indicates the relative potency of the peptide in increasing the current; the number of - signs indicates the relative potency of the peptide in decreasing the current; N E indicates that the peptide has no effect on the current; and x indicates that the peptide has not yet been tested on the current. The responsible authors are mentioned in the introduction as the currents are introduced. The numbers above the currents indicate the references from which the information was obtained. (1) Boyd & Walker, 1985, 1987; Cottrell, Davies & Green, 1984; Cottrell & Davies, 1987; (2) Colombaioni, Paupardin-Tritsch, Vidal & Gerschenfeld, 1985; (3) Ruben, Johnson & Thompson, 1986; (4) Brezina, Eckert & Erxleben, 1987b; (5)Brezina, Eckert & Erxleben, 1987a; (6) Thompson & Ruben, 1988.

Methods

Dissection of the C1 neurone. The cerebral ganglia of Helix aspersa were removed from the animal and pinned out ventral side uppermost in a small Perspex bath lined with a Sylgard base. The bath was filled with normal HEPES saline (80mM NaCl, 5mM KCl, 5mM $MgCl_2$, 7mM $CaCl_2$, 20mM HEPES, pH 7.5). The connective tissue was dissected away with forceps, and with the aid of a dissecting microscope. An agar bridge was incorporated into the circuit because the ionic compositions of the two salines used in this series of experiments were substantially different. The C1 neurone, the largest cell visible on this surface of each ganglion, has a diameter of 100-200μm and so is easily identified. The cell was impaled with a glass electrode filled with 1M CsCl and having a resistance of 1-2 MΩ. The cell was then voltage clamped with a Dagan 8100 single electrode clamp system. Recordings were made on a Racal store 4DS recorder.

Protocol. The membrane potential was held at -50mV, and the bath saline then replaced by Tris barium saline (25mM $BaCl_2$, 75mM TEACl, 10mM KCl, 5mM $MgCl_2$, 3mM 4-AP, 5mM TrisHCl, pH 7.5) (modified from Plant and Standen, 1981). The current flowing through calcium channels was under investigation here and so is referred to as the calcium current, though the actual charge carrier through the calcium channels was barium. The cell was allowed to rest one minute. Voltage steps of 100ms or 1 second duration were applied; first a depolarising step and then an equal hyperpolarising step to record the leakage current. When the currents were later analysed with a computer program designed by Mr. J. Dempster, University of Strathclyde, the leakage currents were subtracted from the calcium currents before

measurements of the currents were made. The cell was depolarised to a maximum membrane potential of +10mV; beyond this value it became difficult to clamp the cell. The steps were applied at 15-20 second intervals so that no voltage step was affected by the previous step. The bath saline was then displaced by one containing the peptide under investigation at the appropriate concentration. The steps were repeated, the peptide was washed away, and the steps repeated again.

Channel blockers. The barium saline contained no sodium so as to prohibit sodium currents. TEACl and 4-AP were added to block the delayed and calcium-dependent potassium currents (Hermann & Gorman, 1981a,b). The potassium A current was blocked by holding the cell at -50mV (this minimises activation) and by the presence of 4-AP in the saline (Thompson, 1977). The calcium-dependent and voltage sensitive potassium currents were blocked by TEACl (Meech & Standen, 1975). Barium is a potassium channel blocker in addition to carrying a larger current through calcium channels than calcium itself (Akaike, Lee & Brown, 1978; Eckert & Lux, 1976). Thus, theoretically, the only current being observed was the inward, voltage-dependent calcium current, though barium and not calcium was the charge carrier.

Verification that the peptides were acting on the calcium current. To test that this was indeed the case, and that the observed net decrease in the inward current was not, in fact, due to an increase in the outward potassium current (FMRFamide also increases the outward potassium conductance in the C1 neurone (Cottrell, Davies & Green, 1984)), the currents were observed in the presence of 10mM cobalt and 50μM cadmium. These are calcium

channel blockers (Akaike, Lee & Brown, 1978). The effects of the peptides on the currents in the presence of these ions were also investigated.

Peptides. pQDPFLRFamide was a gift from Dr. D. Price (Whitney Marine Laboratory, University of Florida). FMRFamide and FLRFamide were purchased from Cambridge Research Biochemicals, YGGFMRFamide from Peninsula Laboratories and the salts in the salines from Sigma or BDH.

Results

FLRFamide and FMRFamide reversibly reduced the inward current (Fig. 27). The threshold for this effect was approximately 2μM; 1μM tetrapeptide was without effect (Fig. 28). The maximum decrease was only 30% of the total calcium current and the peptide inhibited a constant fraction of the current over the range of stepping potentials investigated (Fig. 29). Addition of cobalt and cadmium to the barium saline reduced the inward current to a negligible level and abolished the effect of the peptides (Fig. 30). The three current traces evoked by the voltage step in the presence of cobalt and cadmium were superimposed upon each other indicating that bath application of the peptides had no effect on any current remaining under these conditions. Thus it seems highly probable that the current being investigated was a calcium channel current and that this current was reduced in the presence of FMRFamide and FLRFamide.

pQDPFLRFamide and YGGFMRFamide, however, had no consistent inhibitory effect on the current (Figs. 31, 32). The decrease in the calcium current exhibited no obvious dose-dependency to the tetrapeptides over the concentration range 2-60μM (Table 5).

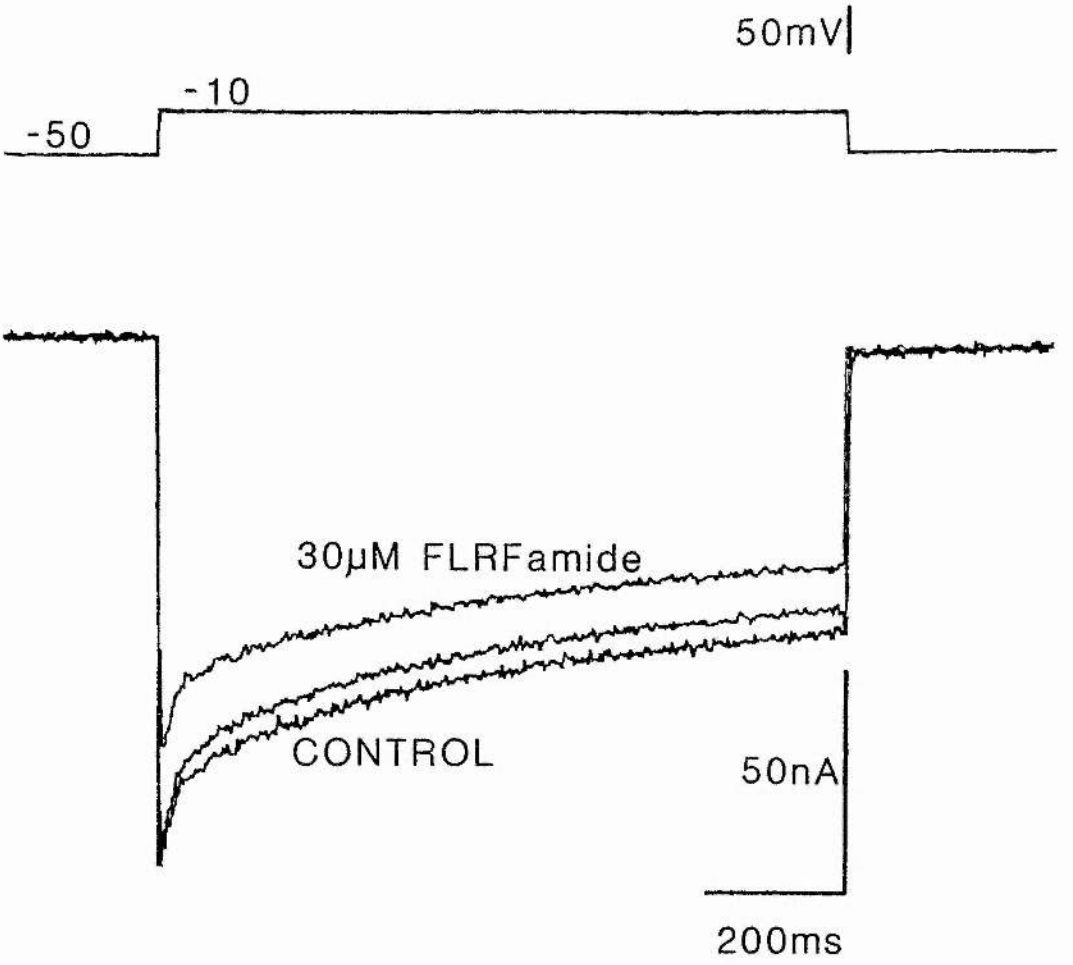


Figure 27. The reversible decrease of the calcium current by bath application of 30µM FLRFamide. The membrane potential was held at -50mV and stepped to -10mV for 1 second.

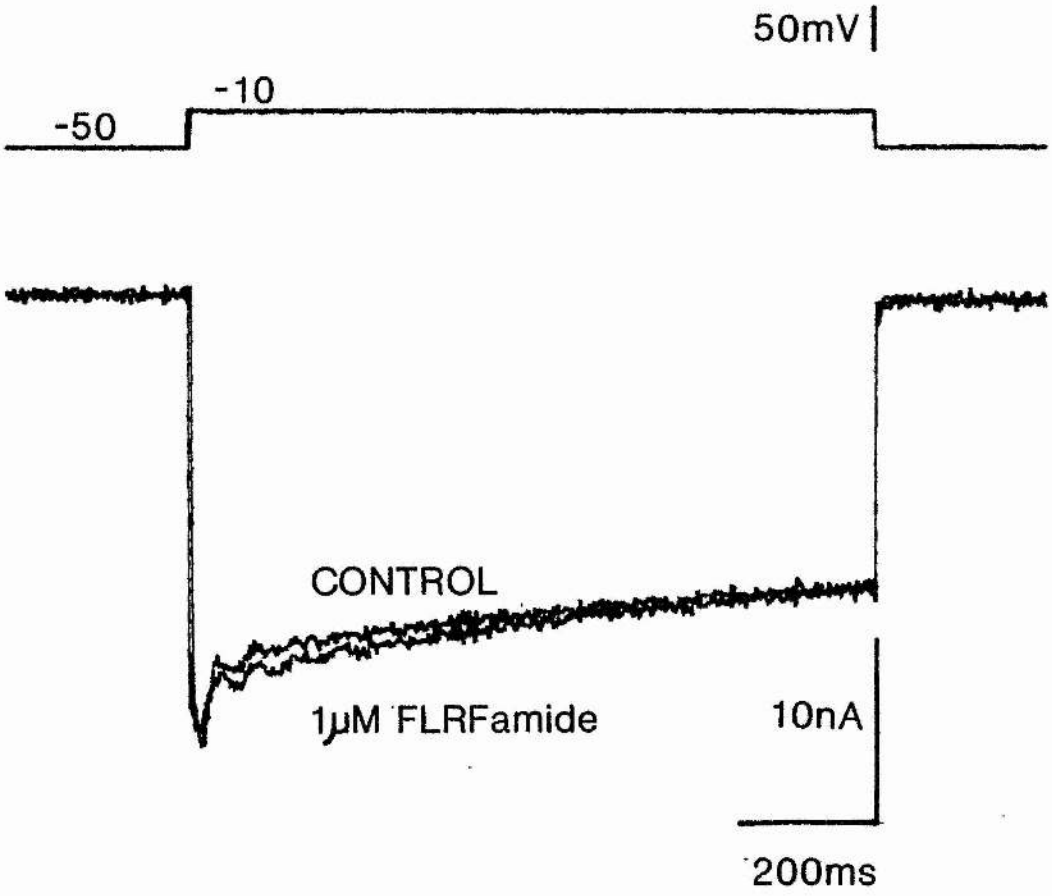


Figure 28. The lack of effect of 1μM FLRFamide on the calcium current. The membrane potential was held at -50mV and stepped to -10mV for 1 second.

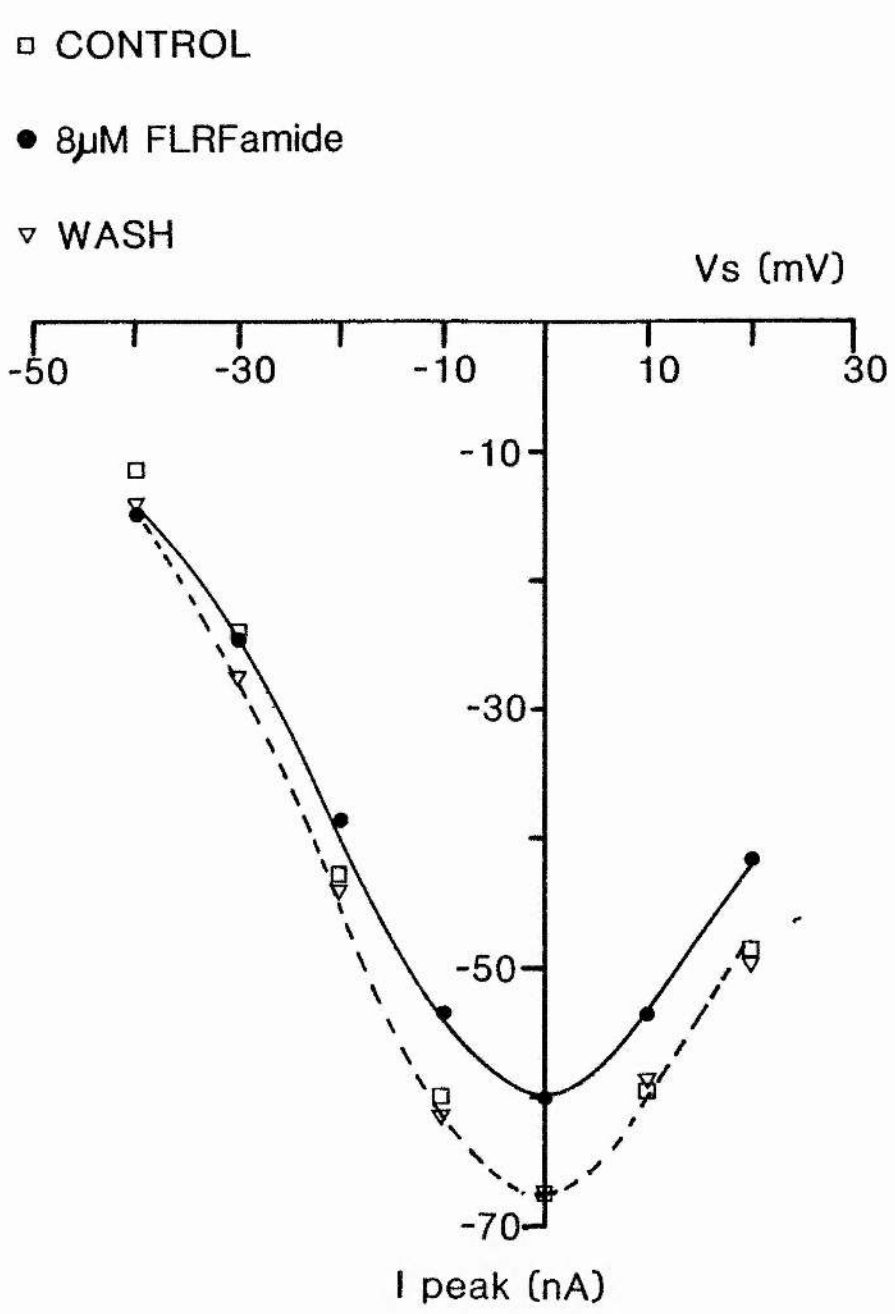


Figure 29. A plot of the peak calcium current evoked by holding the membrane potential at -50mV and stepping to the indicated potentials for 1 second. When the steps are repeated in the presence of 8μM FLRFamide the peak current is reduced but the shape of the curve remains constant.

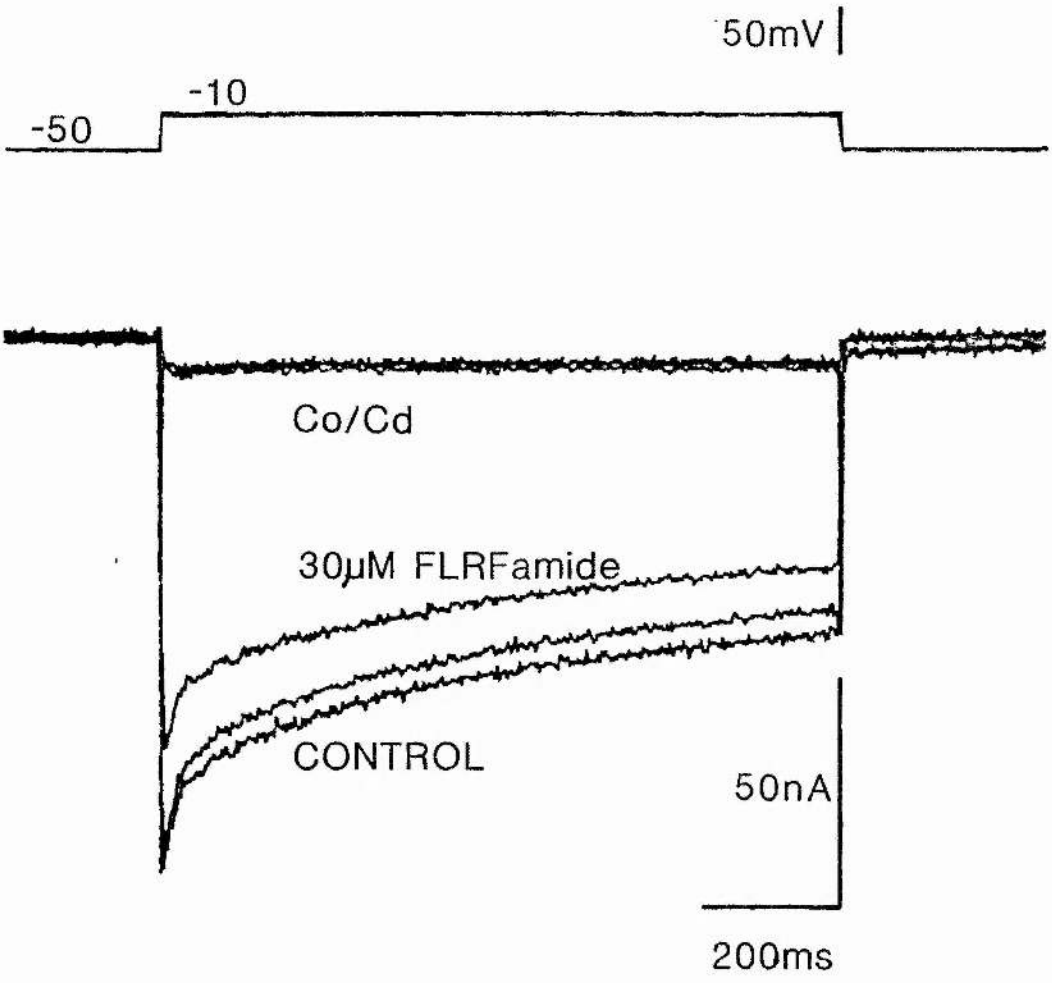


Figure 30. The calcium current was first evoked in the normal barium saline by holding the membrane potential at -50mV and stepping to -10mV for 1 second and was reversibly reduced by the presence of 30µM FLRFamide in the bath. Addition of 10mM $CoCl_2$ and 50µM CdCl reduced the current to a negligible value. FLRFamide was without effect in their presence as well.

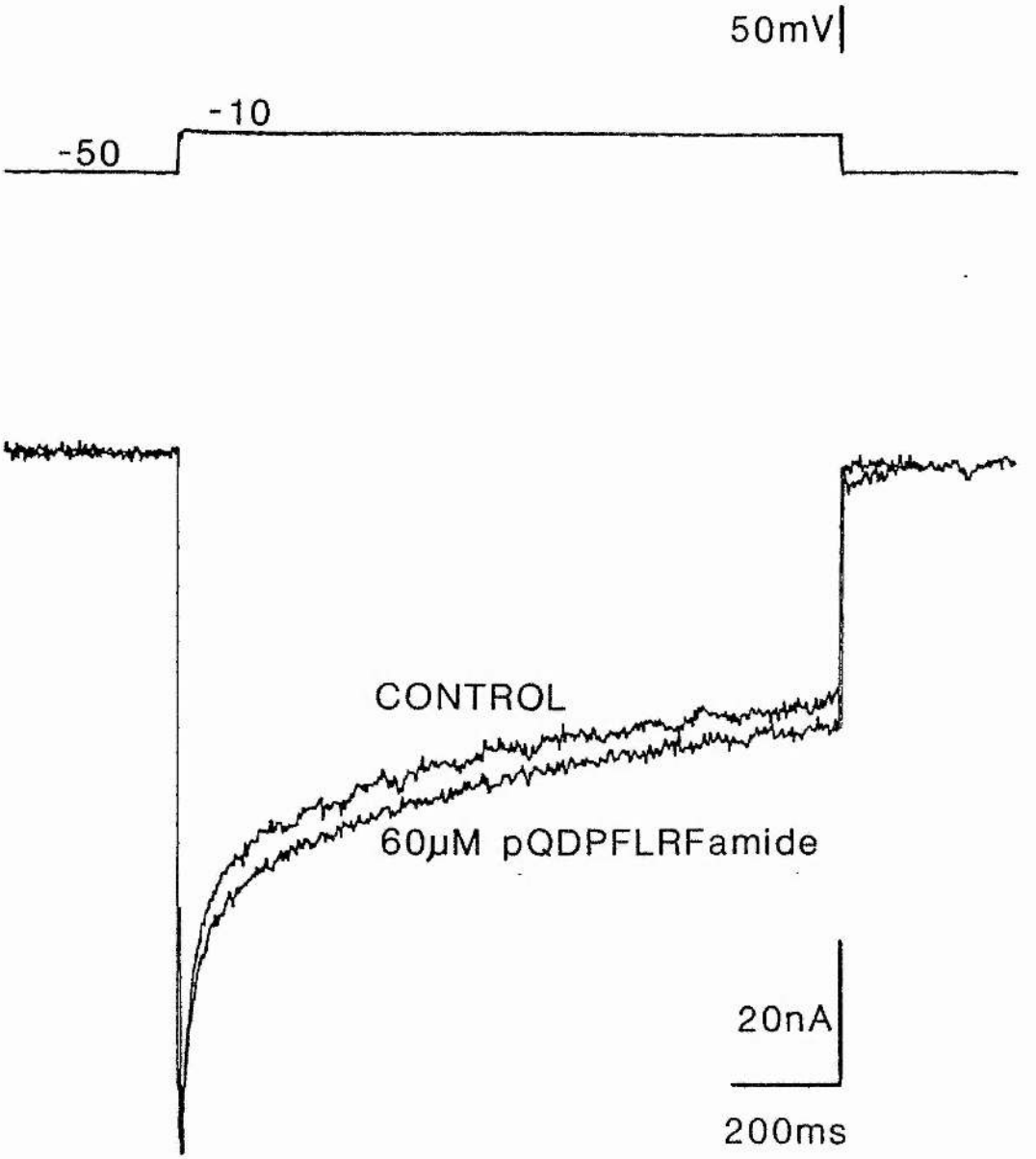


Figure 31. 60µM pQDPFLRFamide did not reduce the calcium current evoked when the membrane potential was clamped at -50mV and stepped to 10mV for 1 second.

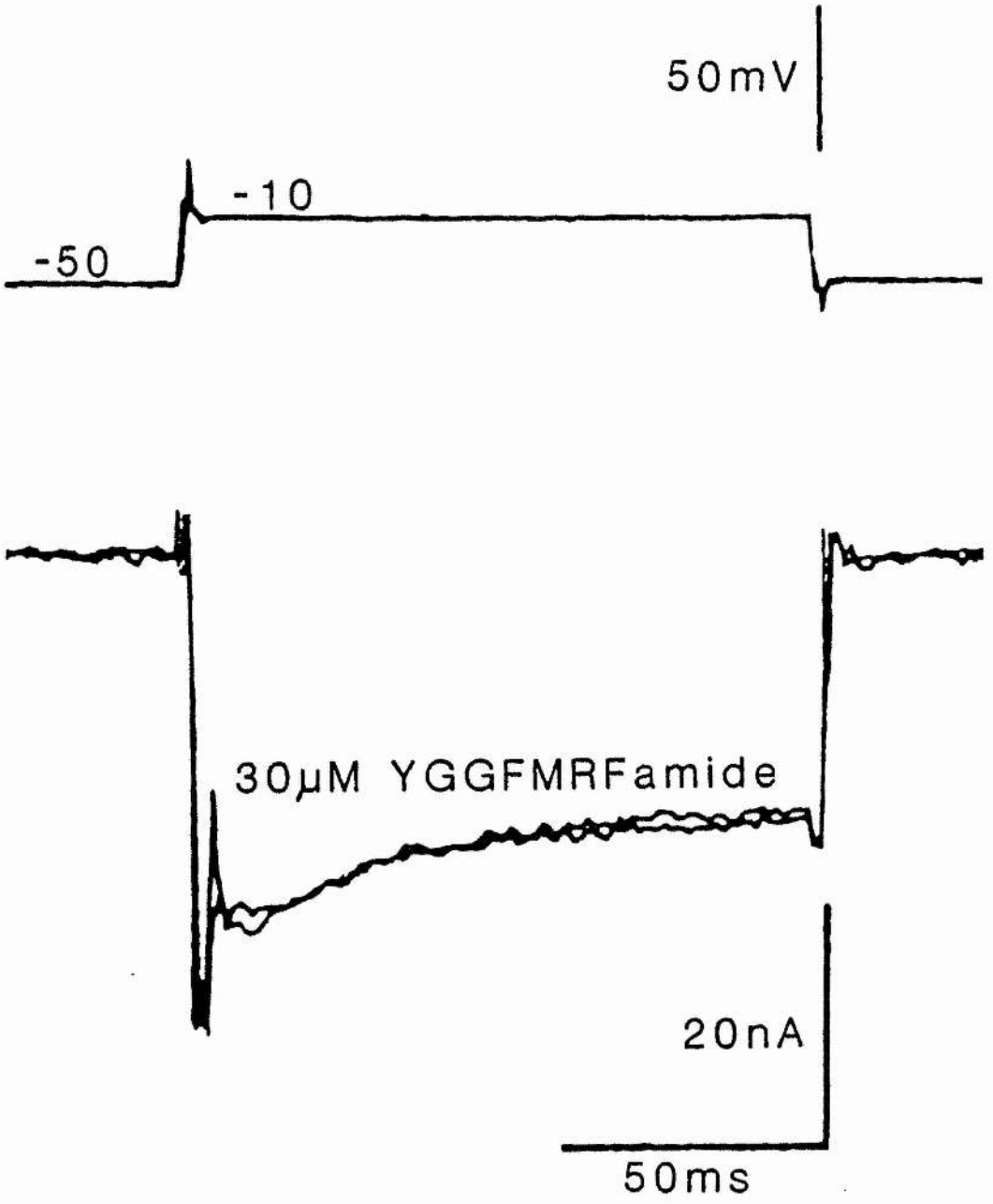


Figure 32. 30µM YGGFMRFamide was without effect on the calcium current evoked by holding the membrane potential at -50mV and stepping to -10mV for 100ms.

% DECREASE (mean ± S.E.M.)

V_H = -50mV V_S = -10mV	1μM	2μM	8μM	15μM	30μM	60μM
pQDPFLRFamide				NONE (2)	NONE (5)	NONE (3)
YGGFMRFamide		NONE (4)			NONE (5)	
FLRFamide	NONE (4)	15.5 ± 2.72 (4)	19.4 ± 5.3 (4)		20.0 ± 2.65 (3)	
FMRFamide	NONE (4)	20.4 ± 2.23 (4)	19.7 ± 3.57 (3)	19 (2)	16.3 ± 6.7 (3)	15.5 (2)

number in brackets = number of experiments

Table 5. A summary of the peptides tested and % decrease (mean +/- standard error of the mean) of the calcium current in their presence at the concentrations indicated. The numbers in brackets are the number of experiments in which each peptide concentration was tested. The membrane potential was held at -50mV and stepped to -10mV for 100ms or 1 second and the peak inward current measured.

Discussion

The ability of the four FMRFamide analogues to decrease the voltage-dependent calcium current seems limited. Only the two tetrapeptides were able to effect a decrease and that was limited to a maximum value of 30%. N-terminal extension of the tetrapeptides to heptapeptide length diminished their potency to zero. A similar effect of extension on the potency of these peptides has been observed for their actions on the sodium current. Table 6 summarises the currents in Helix aspersa neurones which are affected by one or more of the five FMRFamide analogues mentioned in this chapter and the effects of these analogues upon the currents.

	gK slow	gK fast	gNa	gK_V	gCa	gK_S
FMRFamide	+ +	N E	+ +	-	-	- -
FLRFamide	+ +	N E	+ +	-	-	x
FIRFamide	+ +	N E	+ +	-	x	x
pQDPFLRFamide	+	+ +	N E	- -	N E	x
YGGFMRFamide	+	+ +	N E	- -	N E	x

Table 6. A revised table including the effects of FMRFamide, FLRFamide, YGGFMRFamide and pQDPFLRFamide on the various ionic currents investigated in neurones of Helix aspersa. The number of + signs indicates the relative potency of the peptide in increasing the current; the number of - signs indicates the relative potency of the peptide in decreasing the current; N E indicates that the peptide has no effect on the current and x indicates that the peptide has not yet been tested on the current.

FMRFamide modulation of a pacemaker neurone may involve a calcium current. FMRFamide suppresses the beating pacemaker activity of neurone R15 in Aplysia by suppressing a subthreshold (low-voltage-activated, LVA) calcium current (Kramer, Levitan, Carrow & Levitan, 1988). The membrane is hyperpolarised and the

spiking frequency is reduced. This LVA calcium current plays an essential role in generating the bursting pacemaker oscillation and so modulation of the current by FMRFamide inhibits the bursting of the cell. FMRFamide acts directly on the R15 membrane and not indirectly (for example by activating synaptic inputs that inhibit bursting in R15) as isolated juvenile R15 neurones grown in culture respond to FMRFamide in like manner to intact adult neurones.

FMRFamide may inhibit transmitter release by reducing a calcium current. The current-voltage relationship of the C1 calcium current in Helix resembles that of the high-voltage-activated (HVA) current in the B5 neurone of Helisoma trivolvis (Haydon & Man-Son-Hing, 1988). The nonsecretory regions of the B5 neurone exhibit both LVA and HVA calcium currents. However, only the HVA current is present in regions which exhibit secretion. The absence of the LVA current may help to ensure that an action potential is the only stimulus responsible for transmitter release. More recent work on this same system (Haydon, Lukowiak & Man-Son-Hing, 1989) has shown that FMRFamide decreases the magnitude of the HVA current. As FMRFamide decreases the calcium current which is responsible for evoking transmitter release in an identified Helisoma neurone in culture, it is likely that it may also be acting as a modulator of transmitter release in Helix. Thus, if FMRFamide acts at the soma or the terminal of an inhibitory neurone in Helix, by depressing release it can cause apparent stimulation. Conversely, depressing release of an excitatory transmitter would result in apparent inhibition.

Haydon and Zoran (1989) have provided further evidence for this hypothesis. Neurone B5 of Helisoma is a cholinergic neurone which will synapse onto the ACh-sensitive neurone B19 in culture. The release of ACh from B5 can be detected by monitoring the electrical activity of B19. Under control conditions a train of ten electrically-induced action potentials in B5 evoked an average of seven "action potential-evoked postsynaptic potentials" in B19. Addition of 1μM FMRFamide to the bathing medium reduced this number to one and hyperpolarised B5. Washing away the FMRFamide restored the number to an average of seven. Thus, FMRFamide did appear to reduce the quantity of ACh released from the presynaptic terminal.

FMRFamide also affects the secretory apparatus of this system (Man-Son-Hing, Zoran, Lukowiak & Haydon, 1989). The B5 neurone was dialysed with the photolabile calcium cage nitr-5 (loaded to 90% capacity with calcium). Epi-illumination of the cell with UV light released calcium; the increased level of intracellular free calcium evoked release of ACh (measured by monitoring B19 as before). Experiments with fura-2 showed that 1μM FMRFamide did not affect the increased level of free calcium in nitr-5-loaded cells. However, 1-10μM FMRFamide did reduce the calcium-dependent ACh release evoked by photolytic release of calcium from nitr-5. Thus, FMRFamide reversibly decreased the rate of calcium-dependent ACh release under conditions of constant and elevated intracellular free calcium levels. So, in addition to decreasing a presynaptic calcium current required for transmitter release, FMRFamide also seems to reduce the sensitivity of the secretory apparatus to raised internal calcium -- a second mechanism for presynaptic inhibition.

BIBLIOGRAPHY

Abrams, T. W., Castellucci, V. F., Camardo, J. S., Kandel, E. R. & Lloyd, P. E. (1984) Two endogenous neuropeptides modulate the gill and siphon withdrawal reflex in Aplysia by presynaptic facilitation involving cAMP-dependent closure of a serotonin-sensitive potassium channel. Proc. Natl. Acad. Sci. USA 81: 7956-7960.

Agarwal, R. A., Ligon, P. J. B. & Greenberg, M. J. (1972) The distribution of cardioactive agents among molluscan species and tissues. Comp. gen. Pharmac. 3: 249-260.

Agnisola, C., Cariello, L., De Santis, A., Miralto, A. & Tota, B. (1989) Chronotropic and inotropic effects of atrial peptides on the isolated systemic heart of Octopus vulgaris. J. Comp. Physiol. B 158: 637-641.

Akaike, N., Lee, K. S. & Brown, A. M. (1978) The calcium current of Helix neuron. J. Gen. Physiol. 71: 509-531.

Alexandrowicz, J. S. (1964) The neurosecretory system of the vena cava in Cephalopoda. I. Eledone cirrosa. J. mar. biol. Ass. U.K. 44: 111-132.

Alexandrowicz, J. S. (1965) The neurosecretory system of the vena cava in Cephalopoda. II. Sepia officinalis and Octopus vulgaris. J. mar. biol. Ass. U.K. 45: 209-228.

Austin, T., Weiss, S. & Lukowiak, K. (1983) FMRFamide effects on spontaneous and induced contractions of the anterior gizzard in Aplysia. Can. J. Physiol. Pharmacol. 61: 949-953.

Belardetti, F., Kandel, E. R. & Siegelbaum, S. A. (1987) Neuronal inhibition by the peptide FMRFamide involves opening of S K^+ channels. Nature 325: 153-156.

Berry, C. F. & Cottrell, G. A. (1970) Neurosecretion in the vena cava of the cephalopod Eledone cirrosa. Z. Zellforsch. 104: 107-115.

Blanchi, D., Noviello, L. & Libonati, M. (1973) A neurohormone of cephalopods with cardioexcitatory activity. Gen. Comp. Endocrinol. 21: 267-277.

Boer, H. H., Schot, L. P. C., Veenstra, J. A. & Reichelt, D. (1980) Immunocytochemical identification of neural elements in the central nervous systems of a snail, some insects, a fish, and a mammal with an antiserum to the molluscan cardio-excitatory tetrapeptide FMRF-amide. Cell Tissue Res. 213: 21-27.

Boyd, P. J. & Walker, R. J. (1985) Actions of the molluscan neuropeptide FMRF-amide on neurones in the suboesophageal ganglia of the snail Helix aspersa. Comp. Biochem. Physiol. 81C: 379-386.

Boyd, P. J. & Walker, R. J. (1987) Comparison of the effects of FMRF-amide and pQDPFLRF-amide on identified Helix neurons. Comp. Biochem. Physiol. 86C: 371-373.

Brezina, V., Eckert, R. & Erxleben, C. (1987a) Modulation of potassium conductances by an endogenous neuropeptide in neurones of Aplysia californica. J. Physiol. 382: 267-290.

Brezina, V., Eckert, R. & Erxleben, C. (1987b) Suppression of calcium current by an endogenous neuropeptide in neurones of Aplysia californica. J. Physiol. 388: 565-595.

Campanelli, J. T. & Scheller, R. H. (1987) Histidine-rich basic peptide: A cardioactive neuropeptide from Aplysia neurons R3-14. J. Neurophysiol. 57: 1201-1209.

Carroll, L. S., Carrow, G. M. & Calabrese, R. L. (1986) Localization and release of FMRFamide-like immunoreactivity in the cerebral neuroendocrine system of Manduca sexta. J. exp. Biol. 126: 1-14.

Cawthorpe, D. R. L., Rosenberg, J., Colmers, W. F., Lukowiak, K. & Drummond, G. I. (1985) The effects of small cardioactive peptide B on the isolated heart and gill of Aplysia californica. Can. J. Physiol. Pharmacol. 63: 918-924.

Chai, S-H., Tang, J. & Han, J-S. (1986) Antiopioid activity of the cardioexcitatory peptide in central modulation of cardiovascular functions. European J. Pharmacol. 130: 315-318.

Chong, G. C. & Phillis, J. W. (1965) Pharmacological studies on the heart of Tapes watlingi: A mollusc of the family Veneridae. Br. J. Pharmacol. 25: 481-496.

Colombaioni, L., Paupardin-Tritsch, D., Vidal, P. P. & Gerschenfeld, H. M. (1985) The neuropeptide FMRF-amide decreases both the Ca^{2+} conductance and a cyclic 3',5'-adenosine monophosphate-dependent K^{+} conductance in identified molluscan neurons. J. Neurosci. 5: 2533-2538.

Cottrell, G. A. & Davies, N. W. (1987) Multiple receptor sites for a molluscan peptide (FMRFamide) and related peptides of Helix. J. Physiol. 382: 51-68.

Cottrell, G. A., Davies, N. W. & Green, K. A. (1984) Multiple actions of a molluscan cardioexcitatory neuropeptide and related peptides on identified Helix neurones. J. Physiol. 356: 315-333.

Cottrell, G. A., Davies, N. W., Turner, J. & Oates, A. (1988) Actions and roles of the FMRFamide peptides in Helix. In: Neurohormones in Invertebrates. Society for Experimental Biology Seminar Series, vol. 33 (ed. M. C. Thorndyke & G. J. Goldsworthy), pp. 283-298. Cambridge University Press.

Cottrell, G. A. & Lesser, W. (1987) A comparison of the effect of some molluscan (FMRFamide-like) peptides on the calcium current of a snail neurone. J. Physiol. 392: 33P.

Cottrell, G. A. & Osborne, N. (1969) A neurosecretory system terminating in the Helix heart. Comp. Biochem. Physiol. 28: 1455-1459.

Cowden, C., Stretton, A. O. W. & Davis, R. E. (1989) AF1, a sequenced bioactive neuropeptide isolated from the nematode Ascaris suum. Neuron 2: 1465-1473.

Cropper, E. C., Lloyd, P. E., Reed, W., Tenenbaum, R., Kupfermann, I. & Weiss, K. R. (1987) Multiple neuropeptides in cholinergic motor neurons of Aplysia: Evidence for modulation intrinsic to the motor circuit. Proc. Natl. Acad. Sci. USA 84: 3486-3490.

Cropper, E. C., Miller, M. W., Tenenbaum, R., Kolks, M. A. G., Kupfermann, I. & Weiss, K. R. (1988) Structure and action of buccalin: A modulatory neuropeptide localized to an identified small cardioactive peptide-containing cholinergic motor neuron of Aplysia californica. Proc. Natl. Acad. Sci. USA 85: 6177-6181.

Cropper, E. C., Tenenbaum, R., Kolks, M. A. G., Kupfermann, I. & Weiss, K. R. (1987) Myomodulin: A bioactive neuropeptide present in an identified cholinergic buccal motor neuron of Aplysia. Proc. Natl. Acad. Sci. USA 84: 5483-5486.

Dieringer, N., Koester, J. & Weiss, K. R. (1978) Adaptive changes in heart rate of Aplysia californica. J. Comp. Physiol. 123: 11-21.

Dockray, G. J., Reeve, J. R. Jr., Shively, J., Gayton, R. J. & Barnard, C. S. (1983) A novel active pentapeptide from chicken brain identified by antibodies to FMRFamide. Nature 305: 328-330.

Duval, A. (1983) Heartbeat and blood pressure in terrestrial slugs. Can. J. Zool. 61: 987-992.

Ebberink, R. H. M. & Joosse, J. (1985) Molecular properties of various snail peptides from brain and gut. Peptides 6, Suppl. 3: 451-457.

Ebberink, R. H. M., Price, D. A., van Loenhout, H., Doble, K. E., Riehm, J. P., Geraerts, W. P. M. & Greenberg, M. J. (1987) The brain of Lymnaea contains a family of FMRFamide-like peptides. Peptides 8: 515-522.

Eckert, R. & Lux, H. D. (1976) A voltage-sensitive persistent calcium conductance in neuronal somata of Helix. J. Physiol. 254: 129-151.

Erspamer, V. & Ghiretti, F. (1951) The action of enteramine on the heart of molluscs. J. Physiol. 115: 470-481.

Foster, M. & Dew-Smith, A. G. (1875) On the behaviour of the hearts of mollusks under the influence of electric currents. Proc. R. Soc. Lond. XXIII: 318-343.

Froesch, D. & Mangold, K. (1976) On the structure and function of a neurohemal organ in the eye cavity of Eledone cirrosa (Cephalopoda). Brain Res. 111: 287-293.

Frontali, N., Williams, L. & Welsh, J. H. (1967) Heart excitatory and inhibitory substances in molluscan ganglia. Comp. Biochem. Physiol. 22: 833-841.

Fry, H. J. B. (1909) The influence of the visceral nerves upon the heart in cephalopods. J. Physiol. 39: 184-206.

Graff, D. & Grimmelikhuijzen, C. J. P. (1988a) Isolation of <Glu-Ser-Leu-Arg-Trp-NH_2, a novel neuropeptide from sea anemones. Brain Res. 442: 354-358.

Graff, D. & Grimmelikhuijzen, C. J. P. (1988b) Isolation of <Glu-Gly-Leu-Arg-Trp-NH_2 (Antho-RWamide II), a novel neuropeptide from sea anemones. FEBS. Lett. 239: 137-140.

Greenberg, M. J., Painter, S. D. & Price, D. A. (1981) The amide of the naturally occurring opioid [Met] enkephalin-Arg^6-Phe^7 is a potent analog of the molluscan neuropeptide FMRFamide. Neuropeptides 1: 309-317.

Greenberg, M. J., Payza, K., Nachman, R. J., Holman, G. M. & Price, D. A. (1988) Relationships between the FMRFamide-related peptides and other peptide families. Peptides 9, Suppl. 1: 125-135.

Grega, D. S. & Prior, D. J. (1985) The effects of feeding on heart activity in the terrestrial slug, Limax maximus: central and peripheral control. J. Comp. Physiol. A 156: 539-545.

Griffond, B., Boer, H. H. & Wijdenes, J. (1986) Localization and function of an FMRFamide-like substance in the aorta of Helix aspersa. Cell Tissue Res. 246: 303-307.

Grimmelikhuijzen, C. J. P., Dockray, G. J. & Schot, L. P. C. (1982) FMRFamide-like immunoreactivity in the nervous system of hydra. Histochemistry 73: 499-508.

Grimmelikhuijzen, C. J. P. & Graff, D. (1986) Isolation of <Glu-Gly-Arg-Phe-NH_2 (Antho-RFamide), a neuropeptide from sea anemones. Proc. Natl. Acad. Sci. USA 83: 9817-9821.

Grimmelikhuijzen, C. J. P., Hahn, M., Rinehart, K. L. & Spencer, A. N. (1988) Isolation of <Glu-Leu-Leu-Gly-Gly-Arg-Phe-NH_2 (Pol-RFamide), a novel neuropeptide from hydromedusae. Brain Res. 475: 198-203.

Hamill, O. P., Marty, A., Neher, E., Sakmann, B. & Sigworth, F. J. (1981) Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. Pflügers Arch. 391: 85-100.

Haydon, P. G., Lukowiak, K. & Man-Son-Hing, H. J. (1989) FMRF-amidergic modulation of the calcium current responsible for evoking neurotransmitter release in an identified neurone isolated from Helisoma. J. Physiol. 410: 58P.

Haydon, P. G. & Man-Son-Hing, H. (1988) Low- and high-voltage-activated calcium currents: their relationship to the site of neurotransmitter release in an identified neuron of Helisoma. Neuron 1: 919-927.

Haydon, P. G. & Zoran, M. J. (1989) Formation and modulation of chemical connections: Evoked acetylcholine release from growth cones and neurites of specific identified neurons. Neuron 2: 1483-1490.

Hermann, A. & Gorman, A. L. F. (1981a) Effects of 4-aminopyridine on potassium currents in a molluscan neuron. J. Gen. Physiol. 78: 63-86.

Hermann, A. & Gorman, A. L. F. (1981b) Effects of tetraethylammonium on potassium currents in a molluscan neuron. J. Gen. Physiol. 78: 87-110.

Hirata, T., Kubota, I., Takabatake, I., Kawahara, A., Shimamoto, N. & Muneoka, Y. (1987) Catch-relaxing peptide isolated from Mytilus pedal ganglia. Brain Res. 422: 374-376.

Isgrove, A. (1909) Eledone. L.M.B.C. Memoirs. XVIII.

Jacobs, A. A. C., & Van Herp, F. (1984) Immunocytochemical localization of a substance in the eyestalk of the prawn, Palaemon serratus, reactive with an anti-FMRF-amide rabbit serum. Cell Tissue Res. 235: 601-605.

Jaeger, C. P. (1961) Physiology of Mollusca - I. Action of acetylcholine on the heart of Strophocheilos oblongus. Comp. Biochem. Physiol. 4: 30-32.

Jaeger, C. P. (1966) Neuroendocrine regulation of cardiac activity in the snail Strophocheilus oblongus. Comp. Biochem. Physiol. 17: 409-415.

Kavaliers, M., Hirst, M. & Mathers, A. (1985) Inhibitory influences of FMRFamide on morphine- and deprivation-induced feeding. Neuroendocrinology 40: 533-535.

Kempf, S. C., Masinovsky, B. & Willows, A. O. D. (1987) A simple neuronal system characterized by a monoclonal antibody to SCP neuropeptides in embryos and larvae of Tritonia diomedea. (Gastropoda, Nudibranchia). J. Neurobiol. 18: 217-236.

Kerkut, G. A. & Cottrell, G. A. (1963) Acetylcholine and 5-hydroxytryptamine in the snail brain. Comp. Biochem. Physiol. 8: 53-63.

Kerkut, G. A. & Laverack, M. S. (1958) Neurohormone in the snail, Helix aspersa. J. Endocrin. 16: xii-xiii.

Kerkut. G. A. & Laverack, M. S. (1960) A cardio-accelerator present in tissue extracts of the snail Helix aspersa. Comp. Biochem. Physiol. 1: 62-71.

Kerkut, G. A., Sedden, C. B. & Walker, R. J. (1966) The effect of DOPA, α-methyldopa and reserpine on the dopamine content of the brain of the snail, Helix aspersa. Comp. Biochem. Physiol. 18: 921-930.

Klein, M., Camardo, J. & Kandel, E. R. (1982) Serotonin modulates a specific potassium current in the sensory neurons that show presynaptic facilitation in Aplysia. Proc. Natl. Acad. Sci. USA 79: 5713-5717.

Kobayashi, M. (1987) Innervation and control of the heart of a gastropod, Rapana. Experientia 43: 981-986.

Koo, A., Chan, W. S., Ng, W. H. & Greenberg, M. J. (1983) Microvascular vasodilator effect of FMRF-amide and Met-enkephalin-Arg^{6}-Phe^{7}-amide in the rat. Microcirculation 2: 393-412.

Krajniak, K. G., Greenberg, M. J., Doble, K. E. & Price, D. A. (1985) Localization of FMRFamide-related peptides in the slug, Limax maximus, and their effects on the isolated crop and penis. Amer. Zoo. 25: 15A.

Krajniak, K. G. & Price, D. A. (1989) Authentic FMRFamide is present in the Polychaete Nereis virens. Peptides (submitted).

Kramer, R. H., Levitan, E. S., Carrow, G. M. & Levitan, I. B. (1988) Modulation of a subthreshold calcium current by the neuropeptide FMRFamide in Aplysia neuron R15. J. Neurophysiol. 60: 1728-1738.

Kuhlman, J. R., Li, C & Calabrese, R. L. (1985a) FMRF-amide-like substances in the leech. I. Immunocytochemical localization. J. Neurosci. 5: 2301-2309.

Kuhlman, J. R., Li, C & Calabrese, R. L. (1985b) FMRF-amide-like substances in the leech. II. Bioactivity on the heartbeat system. J. Neurosci. 5: 2310-2317.

Le Gall, S., Féral, C., Van Minnen, J. & Marchand, C. R. (1988) Evidence for peptidergic innervation of the endocrine optic gland in Sepia by neurons showing FMRFamide-like immunoreactivity. Brain Res. 462: 83-88.

Lehman, H. K. & Greenberg, M. J. (1987) The actions of FMRFamide-like peptides on visceral and somatic muscles of the snail Helix aspersa. J. exp. Biol. 131: 55-68.

Lehman, H. K. & Price, D. A. (1987) Localization of FMRFamide-like peptides in the snail Helix aspersa. J. exp. Biol. 131: 37-53.

Lehman, H. K., Price, D. A. & Greenberg, M. J. (1984) The FMRFamide-like neuropeptide of Aplysia is FMRFamide. Biol. Bull. 167: 460-466.

Li, C & Calabrese, R. L. (1987) FMRFamide-like substances in the leech. III. Biochemical characterization and physiological effects. J. Neurosci. 7: 595-603.

Lloyd, P. E. (1978a) Distribution and molecular characteristics of cardioactive peptides in the snail, Helix aspersa. J. Comp. Physiol. 128: 269-276.

Lloyd, P. E. (1978b) Neurohormonal control of cardiac activity in the snail, Helix aspersa. J. Comp. Physiol. 128: 277-283.

Lloyd, P. E. (1982) Cardioactive neuropeptides in gastropods. Fed. Proc. 41: 2948-2952.

Lloyd, P. E. (1987) Transport of neuropeptides from central ganglia to muscles involved in feeding in Aplysia. Soc. Neurosci. Abstr. 13: 37.

Lloyd, P. E. & Connolly, C. M. (1989) Sequence of pedal peptide: A novel neuropeptide from the central nervous system of Aplysia. J. Neurosci. 9: 312-317.

Lloyd, P. E., Frankfurt, M., Kupfermann, I. & Weiss, K. R. (1985) Co-localization of the SCPs and FMRFamide to motor neurons innervating Aplysia buccal muscle. Soc. Neurosci. Abstr. 11: 482.

Lloyd, P. E., Kupfermann, I. & Weiss, K. R. (1984) Evidence for parallel actions of a molluscan neuropeptide and serotonin in mediating arousal in Aplysia. Proc. Natl. Acad. Sci. USA 81: 2934-2937.

Lloyd, P. E., Kupfermann, I. & Weiss, K. R. (1985) Two endogenous neuropeptides (SCP_A and SCP_B) produce a cAMP-mediated stimulation of cardiac activity in Aplysia. J. Comp. Physiol. A. 156: 659-667.

Lloyd, P. E., Kupfermann, I. & Weiss, K. R. (1987) Sequence of small cardioactive peptide A: A second member of a class of neuropeptides in Aplysia. Peptides 8: 179-184.

Lloyd, P. E., Mahon, A. C., Kupfermann, I., Cohen, J. L., Scheller, R. H. & Weiss, K. R. (1985) Biochemical and immunocytological localization of molluscan small cardioactive peptides in the nervous system of Aplysia californica. J. Neurosci. 5: 1851-1861.

Lloyd, P. E., Schacher, S., Kupfermann, I. & Weiss, K. R. (1986) Release of neuropeptides during intracellular stimulation of single identified Aplysia neurons in culture. Proc. Natl. Acad. Sci. USA 83: 9794-9798.

Lloyd, P. E. & Willows, A. O. D. (1988) Multiple transmitter neurons in Tritonia. II. Control of gut motility. J. Neurobiol. 19: 55-67.

Longley, R. D. & Longley, A. J. (1985) Immunocytochemical localization of serotonin, FMRFamide, BPP, and SCP_B in nudibranch mollusks. Soc. Neurosci. Abstr. 11: 943.

McFarlane, I. D., Graff, D. & Grimmelikhuijzen, C. J. P. (1987) Excitatory actions of Antho-RFamide, an anthozoan neuropeptide, on muscles and conducting systems in the sea anemone, Calliactis parasitica. J. exp. Biol. 133: 157-168.

Mahon, A. C., Lloyd, P. E., Weiss, K. R., Kupfermann, I. & Scheller, R. H. (1985) The small cardioactive peptides A and B of Aplysia are derived from a common precursor molecule. Proc. Natl. Acad. Sci. USA 82: 3925-3929.

Man-Son-Hing, H., Zoran, M. J., Lukowiak, K. & Haydon, P. G. (1989) A neuromodulator of synaptic transmission acts on the secretory apparatus as well as on ion channels. Nature 341: 237-239.

Marchand, C-R., Wijdenes, J. & Schot, L. P. C. (1982) Localisation par la technique cyto-immuno-enzymologique d'un neuropeptide cardio-excitateur (le F.M.R.F.-amide) dans le collier nerveux péri-oesophagien d'Helix aspersa Müller (Gastéropode, pulmoné, stylommatophore). C. R. Acad. Sc. Paris 294: 39-44.

Martin, R., Froesch, D., Kiehling, C. & Voigt, K. H. (1981) Molluscan neuropeptide-like and enkephalin-like material coexists in octopus nerves. Neuropeptides 2: 141-150.

Martin, R., Froesch, D. & Voigt, K. H. (1980) Immunocytochemical evidence for melanotropin- and vasopressin-like material in a cephalopod neurohemal organ. Gen. Comp. Endocrinol. 42: 235-243.

Martin, R., Froesch, D., Weber, E. & Voigt, K. H. (1979) Met-enkephalin-like immunoreactivity in a cephalopod neurohemal organ. Neurosci. Letts. 15: 253-257.

Martin, R. & Voigt, K. H. (1987) The neurosecretory system of the octopus vena cava: A neurohemal organ. Experientia 43: 537-543.

Meech, R. W. & Standen, N. B. (1975) Potassium activation in Helix aspersa neurones under voltage clamp: a component mediated by calcium influx. J. Physiol. 249: 211-239.

Messenger, J. B., Nixon, M. & Ryan, K. P. (1985) Magnesium chloride as an anaesthetic for cephalopods. Comp. Biochem. Physiol. 82C: 203-205.

Morris, H. R., Panico, M., Karplus, A., Lloyd, P. E. & Riniker, B. (1982) Elucidation by FAB-MS of the structure of a new cardioactive peptide from Aplysia. Nature 300: 643-645.

Muneoka, Y. & Matsuura, M. (1985) Effects of the molluscan neuropeptide FMRFamide and the related opioid peptide YGGFMRFamide on Mytilus muscle. Comp. Biochem. Physiol. 81C: 61-70.

Murphy, A. D., Lukowiak, K. & Stell, W. K. (1985) Peptidergic modulation of patterned motor activity in identified neurons of Helisoma. Proc. Natl. Acad. Sci. USA 82: 7140-7144.

Muske, L. E., Dockray, G. J., Chohan, K. S. & Stell, W. K. (1987) Segregation of FMRF amide-immunoreactive efferent fibers from NPY-immunoreactive amacrine cells in goldfish retina. Cell Tissue Res. 247: 299-307.

Osborne, N. N. (1970) Distribution, localisation and functional sigificance of biologically active monoamines in gastropod molluscs. Ph.D. Thesis, University of St. Andrews.

Painter, S. D. (1982) FMRFamide catch contractures of a molluscan smooth muscle: pharmacology, ionic dependence and cyclic nucleotides. J. Comp. Physiol. 148: 491-501.

Painter, S. D. & Greenberg, M. J. (1982) A survey of the responses of bivalve hearts to the molluscan neuropeptide FMRFamide and to 5-hydroxytryptamine. Biol. Bull. 162: 311-332.

Payza, K. (1987) FMRFamide receptors in Helix aspersa. Peptides 8: 1065-1074.

Pentreath, V. W. & Cottrell, G. A. (1970) The blood supply to the central nervous system of Helix pomatia. Z. Zellforsch. 111: 160-178.

Plant, T. D. & Standen, N. B. (1981) Calcium current inactivation in identified neurones of Helix aspersa. J. Physiol. 321: 273-285.

Price, D. A. (1983) FMRFamide: assays and artifacts. In: Molluscan Neuroendocrinology (ed. J. Lever & H. H. Boer), pp. 184-190. Amsterdam, Oxford, New York: North Holland Publishing Company.

Price, D. A. (1986) Evolution of a molluscan cardioregulatory neuropeptide. Amer. Zool. 26: 1007-1015.

Price, D. A. (1987) The distribution of some FMRFamide- and SCP-related peptides in the Mollusca. In Neurobiology Molluscan Models (ed. H. H. Boer, W. P. M. Geraerts & J. Joosse), pp. 208-214. Amsterdam, Oxford, New York: North Holland Publishing Company.

Price, D. A., Cobb, C. G., Doble, K. E., Kline, J. K. & Greenberg, M. J. (1987) Evidence for a novel FMRFamide-related heptapeptide in the pulmonate snail Siphonaria pectinata. Peptides 8: 533-538.

Price, D. A., Cottrell, G. A., Doble, K. E., Greenberg, M. J., Jorenby, W., Lehman, H. K. & Riehm, J. P. (1985) A novel FMRFamide-related peptide in Helix: pQDPFLRFamide. Biol. Bull. 169: 256-266.

Price, D. A., Davies, N. W., Doble, K. E. & Greenberg, M. J. (1987) The variety and distribution of the FMRFamide-related peptides in molluscs. Zoo. Sci. 4: 395-410.

Price, D. A., Doble, K. E., Lee, T. D. & Greenberg, M. J. (1987) The distribution of FMRFamide-related peptides in the gastropods. Soc. Neurosci. Abstr. 13: 1076.

Price, D. A. & Greenberg. M. J. (1977a) Structure of a molluscan cardioexcitatory neuropeptide. Science 197: 670-671.

Price, D. A. & Greenberg. M. J. (1977b) Purification and characterization of a cardioexcitatory neuropeptide from the central ganglia of a bivalve mollusc. Prep. Biochem. 7: 261-281.

Price, D. A. & Greenberg, M. J. (1980) Pharmacology of the molluscan cardioexcitatory neuropeptide FMRFamide. Gen. Pharmac. 11: 237-241.

Prior, D. J. & Watson, W. H. III. (1988) The molluscan neuropeptide, SCP_B, increases the responsiveness of the feeding motor program of Limax maximus. J. Neurobiol. 19: 87-105.

Prosser, C. L. (1940) Acetylcholine and nervous inhibition in the heart of Venus mercenaria. Biol. Bull. 78: 92-102.

Ransom, W. B. (1884) On the cardiac rhythm of invertebrata. J. Physiol. 5: 261-341.

Reinecke, M., Nehls, M. & Forssmann, W. G. (1985) Phylogenetic aspects of cardiac hormones as revealed by immunocytochemistry, electronmicroscopy, and bioassay. Peptides 6, Suppl. 3: 321-331.

Robb, S., Packman, L. C. & Evans, P. D. (1989) Isolation, primary structure and bioactivity of SchistoFLRF-amide, a FMRF-amide-like neuropeptide from the locust, Schistocerca gregaria. Biochem. Biophys. Res. Comm. 160: 850-856.

Ruben, P., Johnson, J. W. & Thompson, S. (1986) Analysis of FMRF-amide effects on Aplysia bursting neurons. J. Neurosci. 6: 252-259.

Smith, P. J. S. & Hill, R. B. (1987) Modulation of output from an isolated gastropod heart: Effects of acetylcholine and FMRFamide. J. exp. Biol. 127: 105-120.

Stell, W. K., Walker, S. E., Chohan, K. S. & Ball, A. K. (1984) The goldfish nervus terminalis: A luteinizing hormone-releasing hormone and molluscan cardioexcitatory peptide immunoreactive olfactoretinal pathway. Proc. Natl. Acad. Sci. USA 81: 940-944.

Sweeney, D. (1963) Dopamine: Its occurrence in molluscan ganglia. Science 139: 1051.

Tang, J., Yang, H.-Y. T. & Costa, E. (1984) Inhibition of spontaneous and opiate-modified nociception by an endogenous neuropeptide with Phe-Met-Arg-Phe-NH_2-like immunoreactivity. Proc. Natl. Acad. Sci. USA 81: 5002-5005.

Thompson, K. J. & Calabrese, R. L. (1988) Effects of FMRFamide on isolated heart muscle cells of the medicinal leech. Soc. Neurosci. Abstr. 14: 12.

Thompson, S. H. (1977) Three pharmacologically distinct potassium channels in molluscan neurones. J. Physiol. 265: 465-488.

Thompson, S. & Ruben, P. (1988) Inward rectification in response to FMRFamide in Aplysia neuron L2: summation with transient K current. J. Neurosci. 8: 3200-3207.

Trimmer, B. A., Kobierski, L. A. & Kravitz, E. A. (1987) Purification and characterization of FMRFamidelike immunoreactive substances from the lobster nervous system: isolation and sequence analysis of two closely related peptides. J. Comp. Neurol. 266: 16-26.

Twarog, B. M. & Page, I. H. (1953) Serotonin content of some mammalian tissues and urine and a method for its determination. Am. J. Physiol. 175: 157-161.

Van Deijnen, J. E., Vek, F. & Van Herp, F. (1985) An immunocytochemical study of the optic ganglia of the crayfish Astacus leptodactylus (Nordmann 1842) with antisera against biologically active peptides of vertebrates and invertebrates. Cell Tissue Res. 240: 175-183.

Voigt, K. H., Kiehling, C., Froesch, D., Schiebe, M. & Martin, R. (1981) Enkephalin-related peptides: direct action on the octopus heart. Neurosci. Letts. 27: 25-30.

Voigt, K.-H. & Martin, R. (1986) Neuropeptides with cardioexcitatory and opioid activity in octopus nerves. In CRC Handbook of Comparative Opioid and Related Neuropeptide Mechanisms, vol. 1 (ed. G. B. Stefano), pp. 127-138. Boca Raton, Florida: CRC Press, Inc.

Wells, M. J. & Mangold, K. (1980) The effects of extracts from neurosecretory cells in the anterior vena cava and pharyngo-ophthalmic vein upon the hearts of intact free-moving octopuses. J. exp. Biol. 84: 319-334.

Welsh, J. H. (1954) Hydroxytryptamine: a neurohormone in the invertebrates. Fed. Proc. 13: 162-163.

Welsh, J. H. (1971) Neurohumoral regulation and the pharmacology of a molluscan heart. Comp. gen. Pharmac. 2: 423-432.

Welsh, J. H. & Hyde, J. E. (1944) The effects of potassium on the synthesis of acetylcholine in brain. Am. J. Physiol. 142: 512-518.

Willows, A. O. D. & Lloyd, P. E. (1983) Synthetic SCP_B elicits patterned neural feeding activity from the buccal ganglia of Tritonia. Soc. Neurosci. Abstr. 9: 386.

Willows, A. O. D., Lloyd, P. E. & Masinovsky, B. P. (1988) Multiple transmitter neurons in Tritonia. III. Modulation of central pattern generator controlling feeding. J. Neurobiol. 19: 69-86.

Wirsig-Wiechmann, C. R. & Basinger, S. F. (1988) FMRFamide-immunoreactive retinopetal fibers in the frog, Rana pipiens: demonstration by lesion and immunocytochemical techniques. Brain Res. 449: 116-134.

Yanagawa, M., Fujiwara, M., Takabatake, I., Muneoka, Y. & Kobayashi, M. (1988) Potentiating effects of some invertebrate neuropeptides on twitch contraction of the radula muscles of a mollusc, Rapana thomasiana. Comp. Biochem. Physiol. 90C: 73-77.

Yang, H.-Y. T., Fratta, W., Majane, E. A. & Costa, E. (1985) Isolation, sequencing, synthesis, and pharmacological characterization of two brain neuropeptides that modulate the action of morphine. Proc. Natl. Acad. Sci. USA 82: 7757-7761.

Zucker, C. L. & Dowling, J. E. (1987) Centrifugal fibres synapse on dopaminergic interplexiform cells in the teleost retina. Nature 330: 166-168.